Biochemical and Electrophysiological Studies on Adenosine Triphosphatase Activity in the Vertebrate Retina

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine

Robert N. Frank
Yale University
School of Medicine
New Haven, Connecticut
April 1, 1966

This research was supported in part by grant NB-03333 from the Institute of Neurological Diseases and Blindness, U. S. Public Health Service, to Dr. Timothy H. Goldsmith, Department of Biology, Yale University. Part of this work was done under the tenure of a U.S.P.H.S. summer student fellowship administered by the Yale Medical School during the summer of 1964, and another part was conducted during the summer of 1965 under the tenure of a "Fight for Sight" Summer Student Fellowship, No. SF-293, financed by a grant from Burroughs Wellcome & Co. (U.S.A.) Inc., to the National Council to Combat Blindness, Inc., New York City.

The following abbreviations are used in this paper: ATP, CTP, GTP, ITP, and UTP for the triphosphates of adenosine, cytosine, guanine, inosine, and uridine; ADP and AMP, adenosine di- and monophosphates; CTAC, cetyltrimethylammonium chloride; DNP, 2, 4-dinitrophenol; EDTA, the sodium salt of ethylenediaminetetraacetic acid; PCMB, parachloromercuribenzoate; NAD, nicotinamide adenine dinucleotide (formerly, diphosphopyridine nucleotide, or DPN); NADP, nicotinamide adenine dinucleotide phosphate (formerly, triphosphopyridine nucleotide, or TPN).

ACKNOWLEDGMENTS

I owe a very great debt of thanks to Dr. Timothy H. Goldsmith, Associate Professor of Biology, Yale University, for his most generous and continued help, encouragement, and advice during the entire course of these experiments.

I should like to thank Dr. Joseph F. Hoffman, Professor of Physiology, Yale University, for very helpful discussions of the results of this work.

Finally, I should like to thank Mrs. Bette Duff and Mrs. Becky Stanley for technical help.

To the memory of my father

STANTON H. FRANK

(July 21, 1907 - March 21, 1966)

"The memory of the good is an enduring benediction."

--Hebrew saying

Table of Contents

Introduction

In 1963, Scarpelli and Craig at Ohio State University demonstrated by histochemical means, using the electron microscope, that there was extensive nucleoside triphosphatase activity in the frog retina, and that this activity was located in part in the rod and cone outer segments, the photoreceptors of the eye (Scarpelli and Craig, 1963). Shortly thereafter, McConnell and Scarpelli (1963) reported that digitonin extracts of cattle rod outer segments contained not only the visual pigment rhodopsin, but also ATP-hydrolyzing activity which appeared to be light-activated, with an action spectrum similar to the well-known absorption spectrum of rhodopsin. They therefore suggested that rhodopsin was a light-activated ATPase, a finding which could have great significance, since the manner by which the visual pigments translate light energy into nerve impulses is as yet unknown.

The group at Ohio State did not note that in recent years a considerable volume of work has been published concerning a widely distributed ATPase activity which requires magnesium, is further stimulated by sodium and potassium together but not separately, and is inhibited by ouabain and other cardiac glycosides. As I shall discuss, a considerable body of evidence has now accumulated that this enzyme system is of central importance in the

active, linked sodium-potassium transport system in many cell membranes. As opposed to other layers in the retina, very little enzymatic activity has been found in the photoreceptor outer segments when biochemical or histochemical techniques were used to assay for a variety of enzymes. If a transport ATPase system were present there, it could have great importance for the physiology of vision, especially were a light-sensitive visual pigment critical for its activity. Hence, the original purpose of the present research was to re-examine the claim of McConnell and Scarpelli, and in particular to determine if photoreceptor outer segment ATPase activity was of the sodium-potassium activated, glycoside inhibited type. As will be shown, ATPase activity was found in high concentration in the outer segments of the pig and the frog, and this activity was indeed of the type that has been implicated in the active transport of sodium and potassium. However, in contrast to McConnell and Scarpelli's findings, pig outer segment ATPase activity was found to be insensitive to light, and moreover was partially separable from rhodopsin. A report of this work has been published (Frank and Goldsmith, 1965), and identical conclusions, based on somewhat different experiments, were reported while this research was in progress (Bonting et al., 1964).

Since, therefore, the photoreceptor ATPase activity

is not a property of the visual pigments, what is its physiological function? A second set of experiments, utilizing electrophysiological methods, attempted an answer to this question by observing the effect on the electrical activity of the isolated frog retina (the electroretinogram or ERG) of incubation in varying concentrations of cardiac glycosides, and by attempting to show that this effect was directly related to the inhibitory action of glycosides on the active transport of sodium and potassium.

This paper will begin with brief summaries of previous work on enzymes in the photoreceptors, on sodium-potassium activated, glycoside inhibited ATPase activity, and on some recent studies of origins of the components of the vertebrate ERG. This discussion is intended as background for the work to be reported later, and no attempt is made to present a comprehensive review of the literature. Reference is made to recent reviews where these are available.

Enzymatic activity in the photoreceptors: Of special interest in the study of retinal enzymology are the elegant experiments of Lowry and his co-workers (Lowry et al., 1956, 1961). They were able to separate individual layers from the retinas of monkeys and rabbits and, utilizing micro-assay techniques, study the localization

and concentration of a number of enzymes, including lactic dehydrogenase, malic dehydrogenase, aspartic-alpha ketoglutaric transaminase, glutamic dehydrogenase, hexokinase, phosphofructokinase, phosphoglucoisomerase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase. While activities of these enzymes varied from layer to layer of the retina, the rod and cone outer segments were markedly low in activity for all the enzymes studied. Similar techniques, used earlier by Anfinsen (1944), localized cholinesterase activity in the synaptic layers of the retina, but not in the outer segments. Histochemical studies by several workers, looking for a variety of enzymes, have also revealed little activity in the outer segments of the photoreceptors (Cogan and Kuwabara, 1959; Kuwabara and Cogan, 1960; Berkow and Patz, 1961; Niemi and Merenmies, 1961a, 1961b; Eränkö et al., 1961; Pearse, 1961; Lessell and Kuwabara, 1964).

Among those enzymes which appear to be present, as demonstrated by deposition of pigmented reaction products in the outer segment layers of retinal sections examined microscopically following histochemical staining, are the following: beta-hydroxybutyrate dehydrogenase and 6-phosphogluconate dehydrogenase (Pearse, 1961), a variety of phosphatases (Lessell and Kuwabara, 1964), monoamine ox-

idase (Eränkö et al., 1961), and cytochrome oxidase (Niemi and Merenmies, 1961b; Eränkö et al., 1961). The activities of the dehydrogenases and phosphatases (including an ATPase) in these studies were low as compared to activity present in other retinal layers, and it is difficult to determine whether this represents actual presence of these enzymes in the outer segments, or artefact due to deposition of some reaction product in the absence of specific enzymatic activity, or migration of reaction product from enzymatic sites in other retinal layers into the enzymatically barren outer segments. It may also be noted that some of these enzymes apparently present in low activities in the histochemical studies were absent in the biochemical determinations of Lowry et al. Most workers seem agreed that the photoreceptors in most species appear, at least on the basis of present evidence, to be relatively devoid of enzymes (Cogan et al., 1961).

Possible exceptions to this are the reports of Eränkö et al. (1961) of monoamine oxidase activity located in high concentrations by histochemical methods in the outer segments in some species, and the claim by the same group of authors (Eränkö et al., 1961; Niemi and Merenmies, 1961b) that cytochrome oxidase, detected by one of the "Nadi" histochemical reactions, was strongly present in the outer segments. Whether the monoamine oxidase reaction represented true activity of this enzyme is questionable, since

addition of known MAO inhibitors to the reaction medium did not reduce the staining (Eränkö et al., 1961). With regard to cytochrome oxidase, the products of the "Nadi" reactions are known to be strongly lipid soluble (Pearse, 1960), and the outer segments contain a very high proportion of lipid material (Collins et al., 1951, 1952). It would appear likely that Niemi and Merenmies' results were artefact, perhaps caused by creepage of the lipid-soluble reaction product from the reaction site to the lipid-rich outer segments nearby. Some years ago, Ruth Hubbard (1954a) reported oxygen uptake to be high in suspensions of rod outer segments; however, her preparations probably contained significant numbers of mitochondria, so that the presence of respiratory chain enzymes in outer segments is not established.

That this criticism is indeed justified is suggested by a very recent paper by McConnell (1965). He claims to have prepared a very pure outer segment fraction from cattle retinas by differential centrifugation in a sucrose density gradient. The evidence for this is (a) electron microscopy of these preparation reveals only rod discs, membrane fragments, and no mitochondria; (b) all the rhodopsin present was in these fractions; and (c) enzyme assays of these fractions revealed ATPase and ascorbate oxidase activities, along with a high lipid phosphorus content, but no succinoxidase, NADH oxidase, glucose-6-phosphatase,

cytochrome oxidase, or NADPH-cytochrome c reductase activity was present. These findings are interpreted to mean that mitochondrial and microsomal particles were absent from the "outer segment" fractions. These results further limit the range of enzymes that may be present in outer segments, although they by no means argue conclusively for the purity of the "outer segment" preparations of McConnell. The enzymes absent from outer segment fractions were, however, present in other retinal fractions in these experiments.

Enzymes which may be important for the metabolism of rhodopsin in the retina are retinal (retinene) isomerase, discovered by Hubbard (1956), and an alcohol dehydrogenase, which, with NAD as cofactor, oxidizes retinol (vitamin A) to retinal in vitro (Hubbard and Wald, 1951).* Wald and Hubbard (1948-49) were able to demonstrate the presence of "retinene$_1$ reductase" activity, along with a separate enzyme required to reduce the oxidized form of NAD in isolated frog outer segments. NAD (and Futterman (1963) has shown that, in rod outer segments, NADP may also be used) had to be added to Wald and Hubbard's rod outer segment suspensions, however. These results may be due to con-

*By recently adopted international convention (Commission on Nomenclature in Biological Chemistry, 1960), vitamin A_1 alcohol is now known as retinol; the aldehyde, formerly called vitamin A_1 aldehyde, retinaldehyde, or retinene$_1$, is now called retinal, and vitamin A_1 acid is known as retinoic acid. The corresponding derivatives of vitamin A_2 are now known as dehydroretinol, dehydroretinal, and dehydroretinoic acid.

tamination of the outer segment preparations by other cell types, however, since Pearse (1961) found alcohol dehydrogenase histochemically in the pigment epithelium but not in the outer segments of the albino rat.

Retinal isomerase has not yet been localized to a specific site in the retina, but in the frog at least, it may occur in the pigment epithelium (Hubbard, 1956). This enzyme facilitates the interconversion of all-*trans* retinal with the 11-*cis* stereoisomer, which is the form required for visual pigment synthesis.

Futterman (1963) has reported alcohol dehydrogenase (i.e. "retinene reductase"), 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase activity in aqueous extracts of cattle rod outer segments. It is hard to know how to reconcile these studies with those of Lowry *et al.* (1956, 1961) and of Pearse (1961) who, examining other species, found no such activity. In addition to species variations, there seem to be at least two possibilities. Either (i) Futterman's preparations of outer segments were contaminated, which is likely since it is often difficult to prepare outer segment suspensions as homogeneous as, say, a suspension of liver mitochondria, or, less likely, (ii) the technique used by Lowry *et al.*, involving the rapid freezing of whole eyes in liquid nitrogen and then cutting thin sections for assay, may have de-

stroyed some enzymatic activity. It is of note also, that Lowry et al. (1956) found relatively little phospholipid in outer segments. This is in contrast to the findings of a variety of other workers (see, e.g. Collins, et al. 1951, 1952; Hubbard, 1954b) who have used biochemical techniques for assay of phospholipids. It is also apparently contradicted by the fact that Sudan Black B, a well-known histological stain for lipids, colors the outer segments much more intensely than the remaining retinal layers (Sidman, 1958, and personal observations of the author). and the electron microscopic observations (Cohen, 1960, 1963; Sjöstrand, 1961; DeRobertis, 1956) that rod and cone outer segments consist of piles of close-packed, membranous discs, which may certainly be presumed, on the basis of present-day theories of membrane structure, to be rich in phospholipids.

ATPase activity in the retina (DeBerardinis and Auricchio, 1951; Auricchio and DeBerardinis, 1951; Lessell and Kuwabara, 1964) and in the outer segments (Sekoguti, 1960; Lessell and Kuwabara, 1964) has been previously reported, and indeed its stimulation by light has been claimed (Sekoguti, 1960; McConnell and Scarpelli, 1963), however, cation activation was not studied. The response to light obtained by Sekoguti was so slight and so variable that no definite conclusions could be drawn regarding the light-sensitivity of the enzymatic activity. In the McConnell and Scarpelli paper, sufficient experimental detail and

quantitative data are not given to enable the reader to assess the validity of the conclusions.

Aside from enzymes, it is well known that the rod outer segments are rich in phospholipids, amounting to 30-40% of the dry weight of the outer segments in frogs and cattle (Collins et al. 1951, 1952; Hubbard, 1954b), and have a high proportion of their non-lipid dry weight as rhodopsin, estimated at 22% in cattle and 60% in the frog (Hubbard, 1954b). Since, aside from these high concentrations of phospholipid and visual pigment, and, perhaps, some enzymes which do not seem to be present in large quantities, no other constituents of note have been established in the outer segments, the discovery there of high concentrations of an enzyme system that has been strongly implicated in the active, linked transport of sodium and potassium (Skou, 1965) must lead to speculation as to its function in visual physiology.

Sodium-potassium activated ATPases and their presumed role in active cation transport: This section attempts only to summarize briefly the evidence for a central role of these enzyme systems in the active, linked transport of sodium and potassium. Skou has reviewed the role of sodium-potassium activated ATPases in active transport of these ions (Skou, 1965). Judah and Ahmed (1964) have reviewed the recent literature on sodium transport, and

Glynn (1964) has discussed the activity of cardiac glycosides as inhibitors of the sodium-potassium "pump."

The existence of an energy-requiring transport system which "pumps" sodium and potassium ions in opposite directions across cell membranes against concentration gradients has been demonstrated in many tissues, having been studied especially extensively in neural tissue (Hodgkin, 1958), red blood cells (Glynn, 1957a), and frog skin (Ussing, 1954). Understanding of the mechanism of this pump has been increased in the years following Skou's report (Skou, 1957) of enzymatic activity from peripheral nerves of a crab species, which hydrolyzed ATP in preference to other nucleoside phosphates, required magnesium for activity, and was further stimulated by sodium and potassium together but not individually. Further work established that ouabain inhibited that fraction of the activity which occurred when sodium and potassium were added to the magnesium-containing system (Skou, 1960). Since Skou's report, sodium-potassium activated, glycoside inhibited ATPase activity has been described in a variety of tissues, including erythrocyte membranes (Post et al., 1960; Dunham and Glynn, 1961), kidney cortex (Wheeler and Whittam. 1962; Kinsolving et al., 1963; Charnock and Post, 1963a), brain fractions (Hess and Pope, 1961; Deul and McIlwain, 1961; Aldridge, 1962; Järnefelt, 1961, 1962; Yoshida and Fujisawa, 1962) cardiac muscle (Auditore and Murray, 1962),

intestinal mucosa (Taylor, 1962), liver microsomes (Schwartz, 1963), and ciliary body (Cole, 1964; Riley, 1964), among others. Bonting, Simon, and Hawkins (1961) found activity in 29 of 36 tissues of the cat and in three human tissues. Of particular interest to the present study was their report of high activity in lyophilized homogenates of whole cat and human retinas.

Although the evidence comes from studies of red cell "ghosts," it is now generally believed that the sodium-potassium stimulated ATPase activity is a central part of the system for active, linked transport of these two ions across cell membranes. The enzyme activity in all tissues where it has been found is bound to membranes, and both the enzymes and the transport system require Na+ and K+ together, utilize ATP in preference to other phosphate esters, and, at least in red cell membranes, are inhibited by similar concentrations of ouabain (Post et al., 1960). Moreover, the glycosides which are the most effective inhibitors of active transport are also the most effective in inhibiting the hydrolysis of ATP (Glynn, 1957b; Dunham and Glynn, 1961). In a very interesting experiment utilizing reconstituted erythrocyte "ghosts," Whittam (1962) showed that the requirement of the ATPase system for cations was, like that of the Na^+-K^+ "pump," asymmetric, requiring internal sodium and external potassium for optimal acti-

vity. In addition, Tosteson et al. (1960) have demonstrated greater ATPase activity in the erythrocytes of sheep with high intracellular K^+ that in a genetic type with low K^+.

Discussions of the possible mechanism of action of the ATPase system in relation to active cation transport have been given by Hokin and Hokin (1960) and by Judah and Ahmed (1964). The former authors favor a multi-enzyme system associated with the phospholipid components of the cell membrane, and utilizing enzymes known to be active in phospholipid metabolism. Judah and Ahmed prefer a system associated with the phosphoprotein fraction, and discuss the evidence favoring this hypothesis and militating against the Hokins' ideas. Recent experiments by Charnock and Post (1963a, 1963b) and Charnock, Rosenthal, and Post (1963) tend to confirm the hypotheses of Judah and Ahmed. Thus, when ATP^{32} is incubated with Na^+ and Mg^{++} and a membrane fraction from kidney cortex, little ATP is hydrolyzed but the tissue takes up the labeled phosphate. Addition of K^+ to this system strikingly increases the hydrolysis of ATP^{32} and removes the label from the membrane fragments. When labeled Na^+ is added to a system containing Mg^{++} and unlabeled ATP, but no K^+, the tissue once again takes up the radioactive label. Extraction of the tissue shows most of the labeled phosphate to reside in the phosphoprotein, rather than the phospholipid, frac-

tion. This work provides evidence in favor of a system of at least two enzymes, the first located near the inner surface of the membrane and requiring Na^+ to split a phosphate group from ATP and bind it to a particular site. When these sites--and there need not be many--are filled, no more ATP can be split, and no more sodium bound. There is no evidence for ATP hydrolysis at this point because few ATP molecules have been split and no phosphate is free in the medium to be measured. However, the tissue fragments containing the enzyme are now labeled with $P^{32}O_4^{-3}$. Addition of K^+ activates a second enzyme, located near the outer surface of the membrane (or perhaps replaces Na^+ at a site on the first enzyme which has migrated outward or rotated), and causes Na^+ to be released along with a phosphate group. The K^+ could then be carried to the inner surface of the membrane and the process repeated.

While the role of the sodium-potassium "pump" in maintaining the trans-membrane electrical potential in many cells is well known, as well as its function in maintaining the excitability of nerve cells for the conduction of action potentials (Davson, 1964), little has been proposed regarding the biochemical origins of slow potentials, usually of low amplitude, which arise in excitable cells in response to a stimulus and may "generate" spikes, or action potentials (hence the term "generator potential"

(Gray, 1959)). The electroretinogram (ERG) of the vertebrate retina is likely to be, at least in part, a record of such a generator potential. Thus, Granit and Therman (1935) have shown that the so-called b-wave of the vertebrate ERG accelerates spike frequencies in the optic nerve, while the a-wave appears to be inhibitory. Similar results had been previously demonstrated by Adrian and Matthews in the eye of the eel (Adrian and Matthews, 1927). The present work, therefore, attempts to determine if the sodium-potassium activated ATPase system of retinal tissue, i.e. the sodium-potassium "pump," is essential for the formation of the ERG. Since the vertebrate retina is a complex tissue containing many types of cells, it may be helpful here to review briefly present concepts about the origins of the vertebrate ERG.

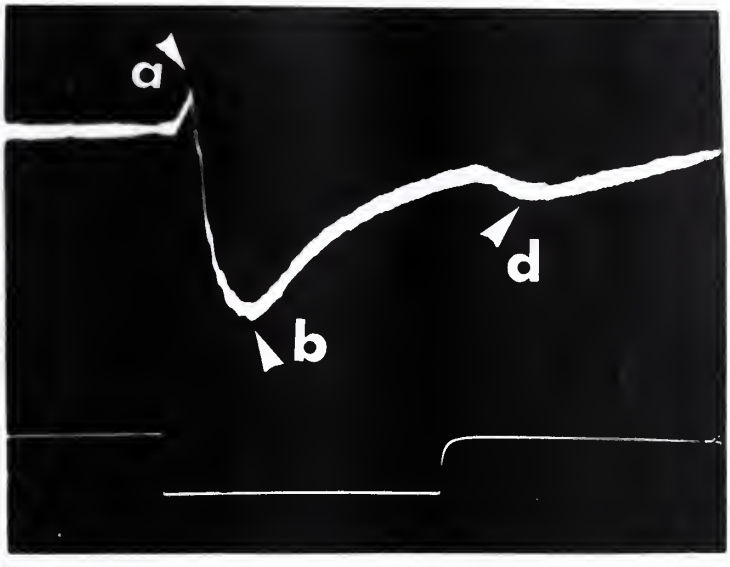
Origins of the ERG in the vertebrate retina: The study of the ERG has been quite detailed and I shall not attempt to review it entirely here. Good recent reviews have been published by Granit (1959, 1962a) and by Brindley (1960), and these include all but some of the recent work which I shall discuss. In the usual recording convention for ERG work (positive deflections up, negative potential deflections down) and with the electrodes as usually placed (recording electrode on the cornea or in the vitreous, reference electrode on the skin or, in the

isolated eye, on the back of the sclera), various deflections in the vertebrate ERG have been labeled the a-, b-, c-, and d-waves (Figure 1). As Brindley points out (Brindley, 1960, pp. 57-58), ERGs from all vertebrates which have been studied are quite similar, except that the d-wave, or off-effect, is more prominent in non-mammalian species. Thus, conclusions drawn from one vertebrate species are clearly applicable to others.

There have been many attempts to assign components of the ERG to various cells of the retina, however, until the recent work of Kenneth Brown and his associates, none of these was thoroughly convincing. Brindley (1960, pp. 58-62) has summarized much of the work until 1960, including microelectrode studies by Tomita, Ottoson and Svaetichin, and by Brindley himself. He points out that, since a microelectrode penetrating the retina from the inside out records no changes in the amplitude of the ERG as the electrode passes through the ganglion cell layer, these cells must contribute nothing to the ERG. This point of view is held as well by Granit (1959), and there is nothing in more recent work to contradict it. The greatest change with distance occurs as the electrode passes the layer containing the bipolar cells, and these cells, in Brindley's view, are the most likely source of the ERG, exclusive of the c-wave. Brindley states

Figure 1

The electroretinogram (ERG) of an isolated frog retina oriented with the receptor surface facing upward, in contact with the recording electrode. In this and all subsequent traces, an upward deflection means that the recording electrode is positive with respect to the reference electrode, and a downward deflection means that it is negative. Hence, all the deflections in this record are mirror images of the way they appear in the usual ERG trace (see text for further discussion). The a-, b-, and d-waves are indicated in the trace. The lower beam indicates the duration of the light stimulus. Note that the d-wave occurs after cessation of the light (off-effect). A c-wave is not present in this record because there is no pigment epithelium present (see text). The c-wave would appear as a prolonged potential of positive polarity, and lasting a considerable period after cessation of the light stimulus.


a
b
d

(Brindley, 1960, pp. 59-60),

> "In particular, there is no feature...which suggests that the a-wave arises in a different layer than the b-wave, or Granit's P II in a different layer from P III (referring to earlier work by Granit which divided the ERG into three components, based on differential effects of drugs, anesthesia, and anoxia). To exclude with certainty such hypotheses involving spatially separated components, simultaneous records of responses to uniform illumination from a number of electrode tracks in the same retina will be required, but published records from single electrode tracks suffice to make them unlikely.... (The) acceptance (of the hypothesis that the bipolar cells are the only structures contributing substantially to the ERG) need not involve assuming that the rods and cones are entirely electrically inactive; but the smallness of the change in recorded response as a microelectrode is moved through the layer of rods and cones certainly implies that the radial extracellular current generated by them, and the difference of potential that it sets up, are very small. The view that the rods and cones are the sole sources of the ERG, is quite untenable now..."

The above discussion refers to the a-, b-, and d-waves of the ERG. As Brindley points out, "the early investigators...all agreed in attributing to the c-wave an origin different from that of the rest of the ERG." The present feeling is that the c-wave, which is a slow, positive potential long outlasting the light stimulus, and readily reduced by light adaptation, originates in the pigment epithelium although it requires the receptors of the retina as well. Noell (1953) found that the c-wave was abolished in the rabbit by intravenous injections of sodium iodate, which destroys the pigment epithelium before attacking other structures in the eye. Yamashita (1959) and Tomita (1959) found the c-wave to be absent in the isolated

amphibian retina and cite this as evidence for its origin in the pigment epithelium, which was not present in their preparations. Brown and Wiesel (1961a) found, using microelectrodes, that the c-wave in the cat retina had maximal amplitude adjacent to the retinal side of Bruch's membrane, and records at this level which appeared to be intracellular had a shape and time course like the c-wave, except, of course, with larger amplitude and reversed polarity. These authors consider it far more likely that these intracellular records were obtained from pigment epithelial cells than from rod or cone outer segments, which were not likely to be penetrated by a microelectrode. Thus, it seems agreed that the c-wave (which is not present in records in the present work, since isolated frog retinas lacking pigment epithelium were used) originates in cells of the pigment epithelium, although the presence of adjacent rod and cone outer segments appears to be necessary as well.

Brown and his co-workers in a series of experiments (Brown and Wiesel, 1961a, 1961b; Brown and Watanabe, 1962a, 1962b; Brown and Murakami, 1964a, 1964b) have shed much light on the origins of the components of the ERG. These authors present strong evidence that Brindley's conclusion, cited above, that the a- and b-waves originate in the same retinal layer, is incorrect. This conclusion was based on microelectrode studies in excised eyes stimulated with a large spot of light, so that the ERG recorded by the micro-

electrode was in fact a summation of potentials throughout the retina recorded at the electrode, but largely originating at foci very distant from the electrode tip. Since the distance between these sites of origin of the potentials and the electrode change very little as the electrode is advanced through the retina, the principal factor determining changes in amplitude will be, not distance (which would be the case if the only cells responding were very close to the electrode track, and no others in the retina responded), but the passive electrical resistance of the medium separating the two electrodes. As this increases, so does the recorded potential. Since the highest resistance in the retina occurs in the central layers (Byzov, 1959), it is not surprising that investigators who recorded ERGs with microelectrodes passing through the retina while stimulating with large spots of light found the amplitudes of all components of the ERG to increase to a maximum at about the layer of bipolar cells, and then remain constant as the electrode penetrated further--since the electrical resistance of the tissue was not significantly increasing. In the experiments of Brown and Wiesel, however, intact eyes in situ in living cats were used, so that there was probably very little retinal tissue that was unresponsive--as was no doubt the case in experiments by other workers, who used excised eyes--and a very small stimulus spot was focused

on the point of entry of the microelectrode into the retina. Since there was very little stray light, the response was truly a local ERG, produced only by cells in the immediate vicinity of the electrode. That this was in fact the case, and that the components of this "LERG" are exactly comparable to the components of the ERG recorded by large electrodes on the cornea or in the vitreous, when the stimulus spot is large, was demonstrated by Brown and Wiesel in an initial paper (Brown and Wiesel, 1961b). This work, as well as later studies, serves to demonstrate that the a-wave originates in the rods and cones, while the b-wave and a slow d.c. component of the same polarity as the b-wave, which had not been identified by previous authors, originate slightly distal to the inner nuclear layer (?in the bipolar cells). The evidence may be summarized as follows:

1. Brown and Wiesel (1961a) show that the amplitude maximum for the d.c. component and the b-wave of the LERG is just distal to the inner nuclear layer, while the peaks of amplitude of the a-wave and the c-wave are just proximal to Bruch's membrane. Allowance is carefully made for the fact that the LERG is in fact an algebraic sum of a-, b-, and c-waves, and a d.c. component of the same polarity as the b-wave. These findings suggest that the a-wave and the c-wave originate very near to Bruch's mem-

brane--and the structures closest to Bruch's membrane are the photoreceptor outer segments proximally and the cells of the pigment epithelium distally--while the site of origin of the b-wave is much more proximal, near the inner nuclear layer.

2. Local anesthetics (e.g. Xylocaine) injected into the retina reduce the b-wave and increase the a-wave (Brown and Wiesel, 1961a). Brown and Wiesel point out that techniques utilized by other workers can also reduce the b-wave independently of the a-wave, but there is no clear evidence that the reverse can occur.* This observation suggests that the a-wave originates in a more distal site (a lower-order neuron) than the b-wave.

3. The a-wave has a shorter latency than the b-wave, which is consistent with a more distal site of origin.

4. Work by A. M. Potts (1962) has demonstrated that, in mouse and rat retinas with intact rod and cone layers but with development of ganglionic and inner nuclear layers impaired by administration of sodium glutamate, the a-wave is normal but the b-wave is reduced or absent.

*The effects of ethanol appear to be an exception to this statement. Thus, Bernhard and Skoglund (1941) found that, when ethanol is applied to the retina of the frog, the b-wave amplitude is greatly increased and its wave form changes, while the a-wave appears diminished (see also results later in this paper). However, as Brown and Wiesel (1961a) point out, the ERG represents the algebraic summation of several components of opposite sign. Thus, if the b-wave (Granit's P II) is increased by some process acting directly on its source, the a-wave (leading edge of Granit's P III), which has the opposite sign, will appear reduced simply because it is partially cancelled by the greatly enlarged b-wave.

5. In monkeys whose ophthalmic artery has been occluded, thus stopping blood flow to the inner retinal layers but not the choroid or the rods and cones (which are supplied by the choroidal arteries), Brown and Watanabe (1962a, 1962b) showed that the a- and c-waves are preserved, but the b-wave disappears. What remains is not simply an a-wave deflection, but a component identical with Granit's P III (Granit, 1959, 1962a), which has the same latency and a leading edge the same shape as the a-wave, but continues as a slow potential of the same sign as that of the a-wave, which either declines abruptly or more slowly when the light is turned off, depending on light intensity, state of dark or light adaptation of the retina, and, in Brown and Watanabe's experiments, on whether the microelectrode was recording from the fovea or more peripherally in the retina.

6. Recordings of the LERG from the foveas of macaque and Cynamolgus monkeys show a- and c-waves, with greatly reduced b-waves, while recordings from other parts of the retinas in these species show complete LERGs (Brown and Watanabe, 1962a). Histologically, these foveas have only cone cells and adjacent pigment epithelium, with other retinal layers swept laterally. When the ophthalmic artery is clamped, only component P III remains, larger in the fovea than in the periphery. In the peripheral retinas

of these monkeys, the P III component has a positive peak at the level of the connecting cilium between the inner and outer segments. As the microelectrode is withdrawn, polarity reverses and reaches a negative maximum at the level of the proximal receptor terminals (receptor-bipolar synapses). This argues for a source of the potential at the ciliary connection and a sink at the proximal receptor terminal. Consistent with this is the fact that, in the fovea, only a positive maximum is recorded at the level of the cone outer segments. No negative potential is seen as the electrode is withdrawn, however the proximal terminals of the foveal cones in these monkeys are swept laterally and hence are not in the electrode path (Brown and Watanabe, 1962a).

7. Mathematical analysis of b-wave amplitude vs. energy of the stimulus light for very low stimulus intensities in the rat eye demonstrates, according to Cone (1963), that the b-wave must originate in second- or higher-order neurons which receive axons from several lower-order cells.

Despite the apparent strength of this evidence, some recent writers disagree. Byzov (1964), who studied the P III component of the frog ERG using microelectrodes, feels that the origin of P III is in the horizontal cells. This author obtained an isolated P III component by applying 2% KCl or 1% tetracaine to the opened frog eye, a procedure which destroys the rest of the ERG. He then found, using a

large stimulus spot, that the amplitude of P III rose as the electrode entered the retina from the vitreous side, reaching a maximum at the outer margin of the inner nuclear layer and changing very little on deeper penetration. Since this is the depth at which the horizontal cells are found, Byzov, largely for this reason, concluded that these cells were the source of P III. However, the same criticism which Brown and Wiesel (1961a) applied to other microelectrode studies using large stimulus spots applies equally well here; indeed, Byzov's results are not very different from those earlier ones of Tomita and of Brindley (1960).

Another dissenter is Hamasaki (1964), who studied the effects on the ERG of the application of various substances, including 10 mM sodium azide, 50 mM KCl, various concentrations of digitonin and formaldehyde, and 5% trypsin, to either the receptor or vitreous surfaces of the isolated frog retina. Most of these substances, applied to the receptor surface, abolished the b-wave within less than a minute, leaving only the isolated P III. This sometimes increased temporarily in amplitude (with 10 mM azide), but in all cases disappeared after about five minutes. Similar events occurred in most cases when these substances were applied to the vitreous surface, but it usually required 10-12 minutes to abolish the ERG. Hamasaki feels that this difference in time course can best be explained by

assuming that all the substances tested act on the receptors, and hence take longer to act when applied to the vitreous surface because of the longer time required to diffuse the greater distance. He feels that this provides evidence that the ERG, exclusive of the c-wave which was not present in his records, is generated entirely by the rods and cones, with the source of the b-wave being simply more labile than the source of the a-wave. While it may be true that the substances Hamasaki tested all acted upon the receptors (and it will be seen that high concentrations of cardiac glycosides act similarly), it does not necessarily follow that (i) the receptors are the only sites of action, and (ii) that the receptors are the source of all the components of the ERG. For example, a receptor potential, the P III component, may arise in a single rod or cone cell after light stimulation and spread proximally to excite a second-order (bipolar) neuron, perhaps by releasing a transmitter substance at the synapse, or by some other means. The second-order neuron could then generate the b-wave. Now, if the receptor-bipolar synapse, or some more distal part of the receptor cell through which the receptor potential had to be conducted to excite the second-order cell, were rendered inactive by a chemical agent, then the b-wave would not occur, although P III would remain.

Evidence for the site of origin of the d-wave, or

off-effect, is less extensive. This component of the ERG was not originally investigated by Brown and Wiesel (1961a), because it is not prominent in the electrical responses obtained from cat eyes. Following later experiments on monkeys, Brown and Watanabe (1962a) stated that the d-wave was most likely the resultant of potentials originating in two different layers of the retina. The initial off-deflection (which has positive polarity in the usual ERG recording) represents the cessation of the negative receptor potential (P III) of the cones. The subsequent negative deflection represents the cessation of the positive d.c. component from the bipolar layer. Brown and Watanabe (1962b) point out that the d-wave does not appear in rod retinas because the receptor potential (P III) in rods is prolonged after cessation of illumination, while that from cones decays promptly.

Brown and his co-workers have also speculated about the nature of the electrical events that give rise to the receptor potential in the rods and cones (Brown and Watanabe, 1962a, 1962b; Brown and Murakami, 1964a, 1964b). In their original experiments, Brown and Wiesel (1961a) reported the positive amplitude maximum for the receptor potential to be located adjacent to Bruch's membrane, that is, it appeared to arise from the distal portions of the outer segments. In later work, Brown and Murakami (1964a) stated that the positive maximum was about 30μ

proximal to Bruch's membrane, in the vicinity of the ciliary connection between the outer segments and the inner segments of the receptors. The negative maximum, as has been stated, was at the level of the receptor-bipolar synapses. This argued for a source of current at the level of the connecting cilium and a sink at the presynaptic membrane. Brown and Murakami state, therefore, that the initial electrical event in the rod, at least the initial event that can be recorded with an extracellular electrode, is a depolarization at the presynaptic membrane, the proximal receptor terminal. This draws current from the level of the connecting cilium. They state that there is no evidence for any electrical change occurring more distally in the outer segments. What this may mean, according to Brown and Murakami, is that the initial photochemical event in the receptor outer segment may be changed into an electric current conducted intracellularly in the ciliary tubules within the connecting cilium and with connections leading to the proximal part of the inner segment. This current may be maximal at the level of the connecting cilium because only there are the intracellular tubules close enough to the cell membrane to be in proximity to an extracellular electrode.

There are, however, a number of difficulties with this theory. First of all, it appears to depend on a misunderstanding of the electron micrographs of Cohen (1960),

on whose work Brown and Murakami have based their anatomical interpretations. Cohen demonstrated tubules originating in the outer segments of the receptors and then running through the connecting cilium to reach a terminus at the basal body in the distal portions of the inner segments. From the basal body arose structures which he called "ciliary rootlets," which, accompanied by vacuolar structures, extended to the proximal inner segments where they ended among the Golgi apparatus. Although Cohen suggested that these structures might indeed have electrical conductive properties, it is clear that some other structure must serve for electrical conduction from the proximal inner segment to the level of the receptor-bipolar synapse. In particular, Cohen demonstrated that, at the level of the cell nucleus, there is only a very thin layer of cytoplasm devoid of organelles between the rod or cone nucleus and the plasma membrane. Hence, at this level and beyond, tubules cannot serve for electrical conduction, which must occur through the nucleus itself, through the cytoplasm, or, more likely, through the plasma membrane--the latter a possibility which Brown and Murakami doubt. But if conduction below the cell nucleus is via the plasma membrane, why should it not occur in this manner distally in the photoreceptor cell as well?

A second difficulty with Brown and Murakami's theory arises from the apparently contradictory results of Hagins,

Zonana, and Adams (1962). These workers excised portions of squid retina and placed fine capillary electrodes proximally and distally along the structure in the squid photoreceptor that corresponds to the vertebrate outer segment. (For details of the fine structure of the cephalopod photoreceptor cells, see Wolken (1958, 1961) and Moody and Robertson (1960).) A very fine beam of light was then focused on the photoreceptor cell, perpendicular to its long axis. When the light was focused on that portion of the cell immediately beneath the tip of one electrode, that electrode went negative with respect to the other. When the light was focused on points intermediate between the electrodes, the electrode nearer the light became briefly negative with respect to the other. At a point midway between the electrodes, the light caused no potential difference between them. Hagins et al. were also able to penetrate the photoreceptor "outer segment" in squid retinas with microelectrodes. When they did so, they found a resting potential difference of 10-50 mv, inside negative. When a fine beam of light was focused on the cell at the site of the penetrating electrode, the electrode recorded a positive deflection with a return to the baseline resting potential. This deflection was of the same form, but opposite sign, as the ones recorded extracellularly. Hagins et al. interpreted their results to mean that light induced a depolarization of the photo-

receptor at the site at which it struck the cell. This depolarization then induced a membrane current which could be conducted along the cell membrane to initiate a neural response.

It will be obvious that Hagins et al. were able to record an electrical response distally in the photoreceptor cell, where Brown and Murakami, in their experiments on vertebrate eyes, were unable to record electrical activity. Second, the response recorded by Hagins et al. was a negative deflection, reflecting depolarization, while Brown and Murakami found a positive deflection at the proximal terminal of the outer segment, at the level of the connecting cilium. There appears to be no good explanation for this latter discrepancy, other than that the mechanism of visual excitation in cephalopod molluscs and in vertebrates may well differ at the receptor level. I believe, however, that there may be a good reason why Brown and Murakami failed to record electrical activity distally in outer segments. In the experiments of Hagins et al., segments of squid retina were excised and a stimulus beam could be directed normal to the long axis of the photoreceptor and at any point along its length. Brown and Murakami, however, used intact monkey eyes, hence, the light beam always entered along the long axis of the receptor cell and always struck the proximal end of the outer segment first. Therefore, maximal light absorption must have occurred at this

end of the photoreceptor, and it is easy to see why the maximal electrical response was always recorded at this point. Had Brown and Murakami been able to direct a light beam normal to the axis of the outer segments, perhaps they would have obtained results similar to those of Hagins et al. and hence, would not have needed to resort to the unlikely hypothesis of intracellular electrical conduction to explain their findings.*

*It is of great interest, though not completely relevant to the work presented here, that Brown and Murakami (1964a, 1964b) have also reported an "early receptor potential" in monkey retinas, having a latency too small to be measured accurately, and presumably arising at the level of the connecting cilium, or perhaps more distally in the photoreceptor cells. This potential is biphasic, having its larger component of the same polarity as the a-wave; it precedes the a-wave in its entirety, and it is obtained only with very high intensity flashes. Brown and Murakami feel that this potential is the direct result of light quanta striking rhodopsin, and if so, it must be the earliest consequence, in electrical terms, of the light stimulus on the retina. Their work has been extended by Pak and Cone (1964) and by Cone (1964a) to several other species, and the shape of the early receptor potential and its latency more clearly determined. Cone (1964a) has provided further evidence, by amplitude vs. stimulus intensity plots, by quantitative studies of the decrease in amplitude of the early receptor potential after exposure to flashes bright enough to bleach away known fractions of the rhodopsin present in the eye, and by a spectral sensitivity curve for the early receptor potential which corresponds to the rhodopsin absorption spectrum, that the early receptor potential is indeed closely related to the effects of light on rhodopsin.

Materials and Methods

Chemicals: (a) Substrates: ATP was supplied as the disodium salt (Sigma Chemical Co., St. Louis, Missouri) or, in some experiments, as the Tris salt (Sigma). Other phosphate esters used were: ADP (Sodium salt, Pabst Laboratories, Milwaukee, Wisconsin), AMP (free acid, Mann Laboratories, New York City), glucose-6-phosphate (Sodium salt, C. F. Boehringer and Son, Mannheim, Germany), GTP, ITP, CTP, UTP (sodium salts, Sigma), and sodium beta-glycerol-phosphate (Fisher Scientific Co., New York City). All substrates were brought to pH 7.2 by addition of Tris before they were added to the reaction mixtures.

(b) Cardiac glycosides: Ouabain was purchased from Sigma and, in all experiments, was dissolved in aqueous media. Scillaren A was purchased from K & K Laboratories, Plainview, New York. Hexahydroscillaren A and cymarin were gifts kindly supplied by Sandoz Pharmaceuticals, Hanover, New Jersey. 17-alpha cymarin was the generous gift of Professor Christophe Tamm of the Institut für organische Chemie, University of Basel, Switzerland. Owing to their limited solubility in aqueous media, these latter four glycosides were initially dissolved in 80% ethanol, which was then diluted to the desired glycoside concentration with the appropriate aqueous medium. Since ethanol is known to have an effect on the ERG (Bernhard and Skog-

lund, 1941), controls were carried out in the electrophysiological determinations, in which retinas were incubated in Ringer's solution containing ethanol without added glycosides.

(c) Extractants of rhodopsin and ATPase activity: Digitonin was obtained from Fisher and was prepared in 2% (w/v) aqueous solution brought to pH 7.2 with Tris. CTAC, obtained from K & K Laboratories, was used in 4% (v/v) aqueous solution brought to pH 7.2 with Tris.

(d) Other chemicals: All other substances used were standard laboratory reagents obtained in the highest commercially available purity.

(e) Animal material: Pig eyes were obtained in New Haven from the Sperry and Barnes Company. The eyes were placed on ice in a light-tight box shortly after enucleation and brought to the laboratory within 24 hours of slaughter.

For experiments requiring frogs, adult leopard frogs (Rana pipiens) of both sexes were used. Frogs used in electrophysiological experiments were obtained in June, July, and August, and frogs used for ATPase assays were obtained in November and December.

Preparation of rod outer segments: Outer segments from pigs were isolated under dim red light by an adaptation of the method of Hubbard (1956). Isolated retinas were

ground lightly in an ice-cold mortar with 40% (w/v) sucrose in 50 mM Tris-HCl buffer, pH 7.2, 0.8 ml per retina. Rod outer segments were detached by forcing the suspension several times through a No. 15 hypodermic needle filed square at the tip. The resulting brei was layered under buffer, and following centrifugation for 10 minutes at 27,000 x g and 4°C., the rod outer segments were collected from the interface and from a U-shaped band on the side of the tube opposite the sedimented retinal debris. The procedure was repeated on the sedimented debris to obtain a second yield of outer segments, and the two batches were pooled.

The outer segments were sedimented by diluting the suspensions threefold with Tris-HCl buffer and centrifuging for 20 minutes at 27,000 x g. The pellet was washed three times by resuspending it in fresh buffer and centrifuging. Finally, the outer segments were lyophilized and stored in the dark at -20°C. In some experiments, the retinal debris (which also contained some attached pigment epithelium) was lyophilized and stored separately in order to compare its enzymatic activity with that of the outer segments.

To prepare outer segments from frogs, three dozen animals at a time were dark adapted for at least one hour, decapitated, and the retinas excised. The remainder of the isolation procedure was carried out in a manner iden-

tical to that just described for pig tissues. Because of the difficulty of dissecting so many small retinas in dim red light, the dissection took place in room lighting. As a result, pigment epithelium migrated into the retinal layers after the prolonged exposure, and separation of retina and pigment epithelium was incomplete. Hence, the inner retinal preparations were contaminated with considerable pigment epithelial tissue, which sedimented with the inner retinal cells during the differential centrifugation step. Outer segments, with a much lower specific gravity, were prepared quite free of contaminating pigment epithelium.

Preparations of pig and frog retinal tissues made in this way retained their enzymatic activity undiminished for at least several weeks.

Preparation of pig liver mitochondria: Pig liver was obtained fresh from the Sperry and Barnes Company and was brought on ice to the laboratory. Fragments of liver were homogenized in 0.25 M sucrose until microscopic examination revealed few intact cells. Mitochondria were separated from the remainder of the homogenate by differential centrifugation, following the method of Schneider (1948). After washing, the mitochondria were lyophilized and stored at -20°C.

Extraction of rhodopsin and ATPase activity: In some

experiments, lyophilized rod outer segments were suspended in either 2% aqueous digitonin or 4% aqueous CTAC. Suspensions contained 10 mg outer segments per ml of CTAC or digitonin. After extraction for 90 minutes in the dark at 4°C., the suspensions were centrifuged for 10 minutes at 27,000 x g and the supernatants were decanted. The sedimented outer segments were washed once in distilled water and resuspended in distilled water. Portions of digitonin or CTAC supernatant, resuspended rod outer segment sediment, and control suspensions of rod outer segments in water, and of rod outer segments suspended in digitonin or CTAC were incubated concurrently, then assayed for enzymatic activity as described below. Absorption spectra were measured in a Cary Model 14 recording spectrophotometer, and rhodopsin concentration estimated from the difference in extinction at 500 mμ, measured before and after bleaching in white light in the presence of 0.1 M hydroxylamine, pH 6. Samples were bleached until no further changes could be observed in the absorption spectra.

Assay of enzymatic activity: The reactions were carried out in a total volume of 0.75 ml. All reaction mixtures contained 0.1 mM EDTA and 1 mM cysteine. Magnesium was added in all experiments in concentration equal to that of ATP. Preliminary experiments showed this ratio

of magnesium to ATP was required to give optimal activity, in accord with reported findings on sodium-potassium stimulated ATPases from other tissues (Skou, 1960; Dunham and Glynn, 1961; Wheeler and Whittam, 1962).

The enzyme preparation was added as a suspension, prepared by adding a weighed quantity of lyophilized tissue into a glass homogenizer tube, adding distilled water (1.0 ml per 5.0 mg dry weight of pig retinal tissue and 1.0 ml per 10.0 mg dry weight of frog tissue), and homogenizing with a few strokes of a teflon pestle. One-tenth ml of this suspension was added to each reaction tube.

Ouabain was added in aqueous solution and other glycosides were added in ethanolic solutions. To reach the glycoside concentration of 10^{-4}M, which was used in the electrophysiological experiments, 4% (v/v) ethanol was required. This concentration was found to inhibit ATPase activity significantly when measured <u>in vitro</u> and hence to reduce the sensitivity of the enzyme assay. Hence, for enzymatic experiments in which glycosides other than ouabain were used, it was elected to use 0.4% ethanol, which was not inhibitory to the enzyme system. This permitted a maximum glycoside concentration of 10^{-5}M for enzyme assays.

The reactions took place in the dark in a 37°C. water bath for the appropriate times. Some experiments involved incubation for 5 minutes, others 30 minutes. Although in

the latter case, rates of reaction were found to decrease due to utilization of substrate in experiments involving pig retinal tissues, the conclusions drawn are not critically dependent on maximal reaction rates.

Reactions were terminated by addition of ice-cold 10% (w/v) trichloroacetic acid. 3.75 ml were added to terminate reactions using pig retinal tissue, and 1.50 ml were used in frog outer segment experiments. This produced a final volume of 4.50 ml in the former case, and 2.25 ml in the latter. By halving the final volume and doubling the quantity of enzyme added, the sensitivity of experiments utilizing frog retinal tissues was increased by a factor of four. This was necessary because frog outer segments were found to have approximately half the total ATPase activity, and one-third the sodium-potassium dependent activity per unit dry weight of tissue, that was present in the same tissue in pigs.

Following addition of trichloroacetic acid all samples were centrifuged, and a 1.0 ml aliquot of the supernate was assayed for inorganic phosphate by the addition of an equal volume of 1% ammonium molybdate in 1.15 N H_2SO_4, to which $FeSO_4$ (4.0 gm/100 ml) had been added shortly before use. The optical density of the final solution was measured at 700 mμ against a distilled water blank within 2 hours of preparation of the color reagent. Concentrations of phosphate were estimated from a standard

curve. In all experiments, controls with no enzyme were done for each substrate, in order to correct for non-enzymatic hydrolysis.

Electrophysiological experiments: Frogs were dark adapted overnight, decapitated, and the retinas carefully dissected out under low-intensity room light, as described by Hamasaki (1963). The total exposure to room light during dissection was less than ten minutes, and, because of this as well as the low intensity, it is doubtful that much visual pigment was bleached during the procedure (Dowling, 1963). Retinas were mounted, usually with the receptor surface oriented upward, on small pieces of filter paper, and placed on a perforated platform in a chamber similar to that described by Hamasaki (1963). Because of this orientation of the retina, ERGs had positive a-waves (upward deflections positive in all recordings) and negative b- and d-waves, just the reverse of their usual appearance when the retina is in the anatomical position, and the recording electrode, on the cornea or in the vitreous, faces the vitreous surface.

The chamber was filled to the level of the upper surface of the platform with frog Ringer of the composition used by Hamasaki (1964), 110 mM NaCl, 2.5 mM KCl, 2.2 mM $CaCl_2$, 6 mM $NaHCO_3$, and 20 mM dextrose. Oxygen was constantly bubbled into the solution during the course of an experiment. The chamber containing the retina was placed

in an electrostatically shielded cage into which no light could enter save the stimulus flash. All experiments were conducted in an air-conditioned room where the temperature was relatively constant at 25 C.

The recording electrode was a silver wire coated with silver chloride, prepared in the same way as that described by Hamasaki (1963). The wire was 0.17 mm in diameter and had a right-angle bend 1-2 mm from the tip. This portion of the electrode made light contact with the upper surface of the retina.

The reference electrode, whose cotton-wick tip was placed in the Ringer bath, was a glass tube filled with frog Ringer, with a cotton wick moistened with Ringer inserted in the lower end and a chlorided silver wire entering through the top opening. Turbulence from the stream of oxygen did not affect the traces, and the photoelectric effect of the stimulus light on the electrodes was negligible.

The electrodes were led into a Type DS2C amplifier (Bioelectric Instruments, Yonkers, N.Y.) and then into a dual-beam oscilloscope. All stages were direct-coupled. The neutralized capacity input circuit of the preamplifier was not employed. For calibration signals, a Bioelectric Instruments Type CA-2 calibrator was connected into the electrode circuit in series with the preparation. A photoelectric cell was connected to the lower input cir-

cuit of the oscilloscope to record the duration of the stimulus light. Responses were observed on the oscilloscope screen, and could be photographed with a Tektronix Type C-12 oscilloscope camera, using Polaroid film.

The light source was a tungsten-filament microscope lamp. The intensity of the stimulus flash, measured at the level of the preparation with a photocell light meter, was approximately 30 lm/m^2. A solenoid-operated camera shutter was opened after a predetermined delay from the start of the oscilloscope sweep, admitting the stimulus flash to the preparation. Since both the light beam and the plane of the retina were horizontal, the stimulus flash was directed onto the retina by an obliquely oriented mirror. The entire retina was uniformly illuminated by the flash, which had a duration of one second.

After dissection and mounting in the chamber, the retina was permitted to re-adapt to darkness for 10-20 minutes. ERGs recorded at this time were always significantly larger than those seen immediately after preparation of the retina. This was, no doubt, the "neural" type of dark adaptation described by Dowling (1963). All components seen in the normal frog ERG were present, except for the c-wave, since, of course, the pigment epithelium was absent. The d-wave, or off-effect, was usually seen immediately after mounting the retina, but disappeared or occurred with very low amplitude subsequently. Since a

large d-wave depends on a state of relative light-adaptation and a high-intensity stimulus, among other things (Granit, 1959), its lack of prominence in these experiments was no doubt due to the dark-adapted state and infrequent stimuli of relatively low intensity that were used. It may be noted, incidentally, that in the earlier experiments of Hamasaki (1963), in which low-intensity stimuli were given only once per minute, d-waves were quite small in his records. In later experiments (Hamasaki, 1964), in which a one-second flash of unspecified intensity was given every five seconds, d-waves appear much more prominently.

After the retina had fully dark-adapted, a control ERG was recorded. Five drops of the test solution were applied directly to the upper surface of the retina, and ERGs were recorded every minute for the first 5-10 minutes, then usually every five minutes thereafter for the duration of the experiment. Every five minutes, the retina was immersed in fresh Ringer, which was then drained to the level of the top of the platform, just wetting the filter paper, and five drops of fresh test solution were dripped onto the retina.

Results

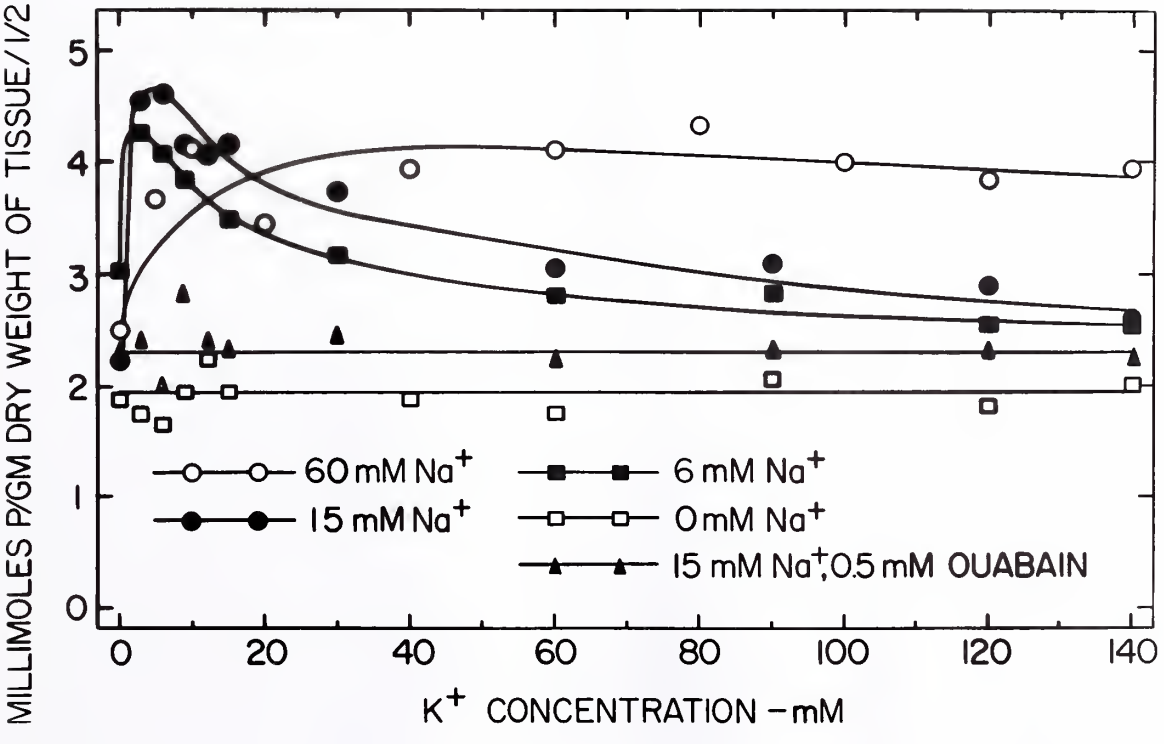
I. Enzymatic experiments on pig retinal tissue

Effects of variation in the concentrations of sodium and potassium: In the presence of 3 mM Mg^{++} and an initial ATP concentration of 3 mM, ATP was hydrolyzed at a relatively low rate. The addition of sodium caused an increase in the rate of about 20%, regardless of the sodium concentration (6-120 mM). Such a slight stimulation by sodium alone has also been noted by Skou (1957, 1960) in his ATPase preparation from crab nerve. The addition of potassium in the absence of sodium had no effect (Figure 2, open squares).

At constant sodium concentrations, addition of potassium ions produced a sharp stimulation of activity (Figure 2). At sodium concentrations of 6-15 mM, the concentration of potassium for half-maximal activity was 1.5 mM; in the presence of 60 mM sodium this figure rose to 4 mM K^{+}. High concentrations of potassium caused an inhibition of activity which could be partially offset by increasing the sodium concentration (Figure 2). In the presence of 100 mM sodium, 6 mM magnesium, and 3 mM ATP, Skou (1960) found the concentration of potassium required for half-maximal activity of crab nerve ATPase was 1.8 mM. Similarly, an inhibition of activity at high

Figure 2

Enzymatic activity of pig rod outer segments as a function of potassium concentration in the presence of constant concentrations of sodium. Medium contained approximately 0.7 mg tissue/ml, 3 mM magnesium, 3 mM Tris-ATP, 0.1 mM EDTA, 1 mM cysteine, and 50 mM Tris buffered to pH 7.2 with HCl. Incubated in darkness for 30 minutes at 37°C.


MILLIMOLES P/GM DRY WEIGHT OF TISSUE/1/2 HOUR
0
1
2
3
4
5
0
20
40
60
80
100
120
140
K+ CONCENTRATION – mM
60 mM Na+
15 mM Na+
6 mM Na+
0 mM Na+
15 mM Na+, 0.5 mM OUABAIN

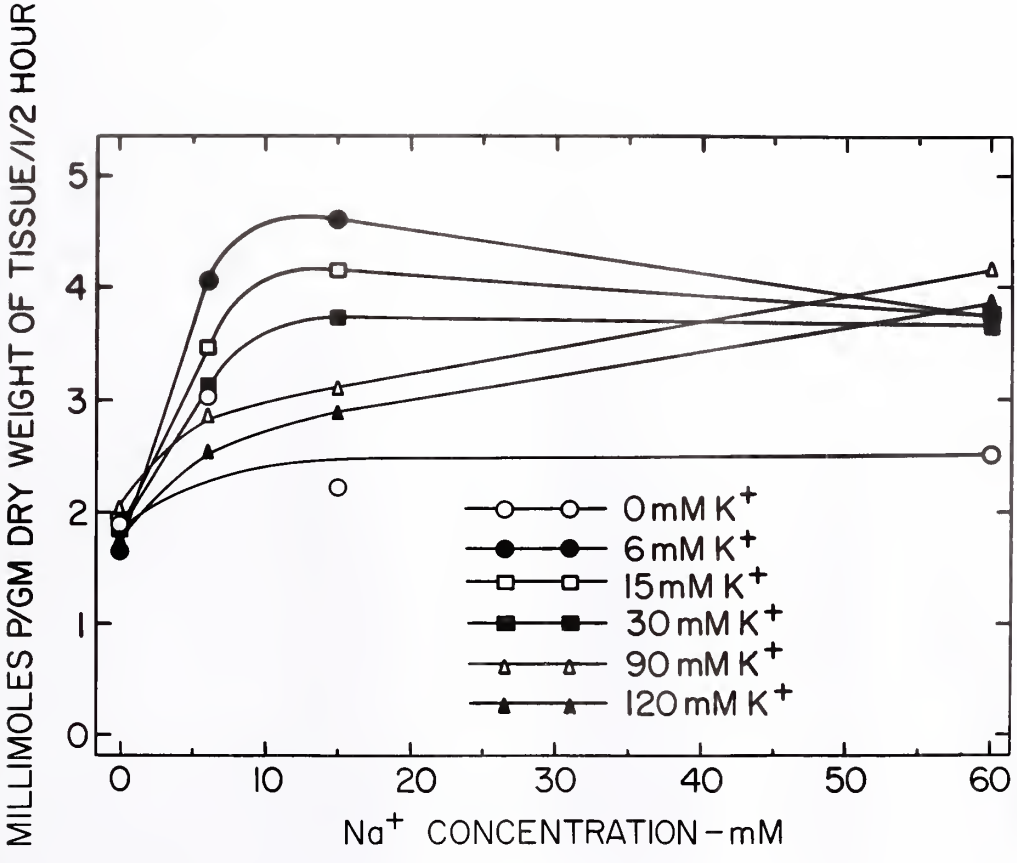
potassium concentrations has been observed with preparations from crab nerve (Skou, 1957) and kidney cortex (Wheeler and Whittam, 1962).

Figure 3 shows the effect of varying the sodium concentration in the presence of constant potassium. Addition of sodium produced an increased rate of hydrolysis until a maximum was reached. At relatively low potassium concentrations, further increases in sodium caused a slight inhibition. Moreover, as the potassium concentration was increased, the concentration of sodium required for half-maximal activity also rose. Thus, at 6 mM potassium, 2 mM sodium produced half-maximal activity, whereas at 120 mM potassium, 32 mM sodium was required. Similar results were reported by Skou (1957), Post et al. (1960), and Auditore and Murray (1962) with ATPase preparations from different sources.

Inhibition of ATPase activity by ouabain: The addition of a sufficient concentration of ouabain to a medium containing enzyme, ATP, magnesium, sodium, and potassium produced inhibition of activity to the level observed when only magnesium and sodium, but no potassium, were present (Figure 2). The concentration of ouabain necessary for half-maximal inhibition was 6.3×10^{-7}M, similar to the values of 3×10^{-7}M obtained by Bonting et al.(1961) for cat ciliary body preparations, and 1×10^{-7}M obtained

Figure 3

Enzymatic activity of pig rod outer segments as a function of sodium concentration in the presence of constant concentrations of potassium. Experimental conditions as described in Figure 2.


MILLIMOLES P/GM DRY WEIGHT OF TISSUE/1/2 HOUR
0
1
2
3
4
5
0
10
20
30
40
50
60
Na+ CONCENTRATION – mM
0 mM K+
6 mM K+
15 mM K+
30 mM K+
90 mM K+
120 mM K+

by Post et al. (1960) with human erythrocyte ATPase, but differing by a factor of more than 30 from the figure of 2×10^{-5}M obtained by Wheeler and Whittam (1962) for rabbit kidney cortex. The effects of various ouabain concentrations on the ATPase activity of pig rod outer segments are plotted in Figure 4.

Effects of pH: Activity was measured over the pH range 6.0-8.5 using 50 mM Tris - 50 mM imidazole, buffered with HCl to the appropriate pH (Figure 5). pH was determined both before and at several times during the course of an incubation. The activity curve with no inhibitor present shows a rather broad maximum at about pH 7.2. With 0.5 mM ouabain present the peak is at approximately the same place, but there is little decrease in activity as the pH increases. The curve for uninhibited activity may therefore be considered the sum of ouabain-sensitive and ouabain-insensitive components with somewhat different shapes.

Enzyme activity with various substrates: Hydrolytic activity was tested using nine different phosphate esters as substrates (Table I). Activity was measured both in the absence of inhibitors and with 0.5 mM ouabain. It is clear that in both the presence and absence of ouabain ATP is the preferred substrate. Adenosine diphosphtate was also hydrolyzed, but largely by the ouabain-insensitive

Figure 4

Enzymatic activity of pig rod outer segments as a function of ouabain concentration. Medium contained approximately 0.7 mg tissue/ml, 3 mM magnesium, 3 mM ATP, 60 mM Na^+, 20 mM K^+, 1 mM cysteine, 0.1 mM EDTA, and 50 mM Tris buffered to pH 7.2 with HCl. Incubated in darkness for 30 minutes at 37°C.

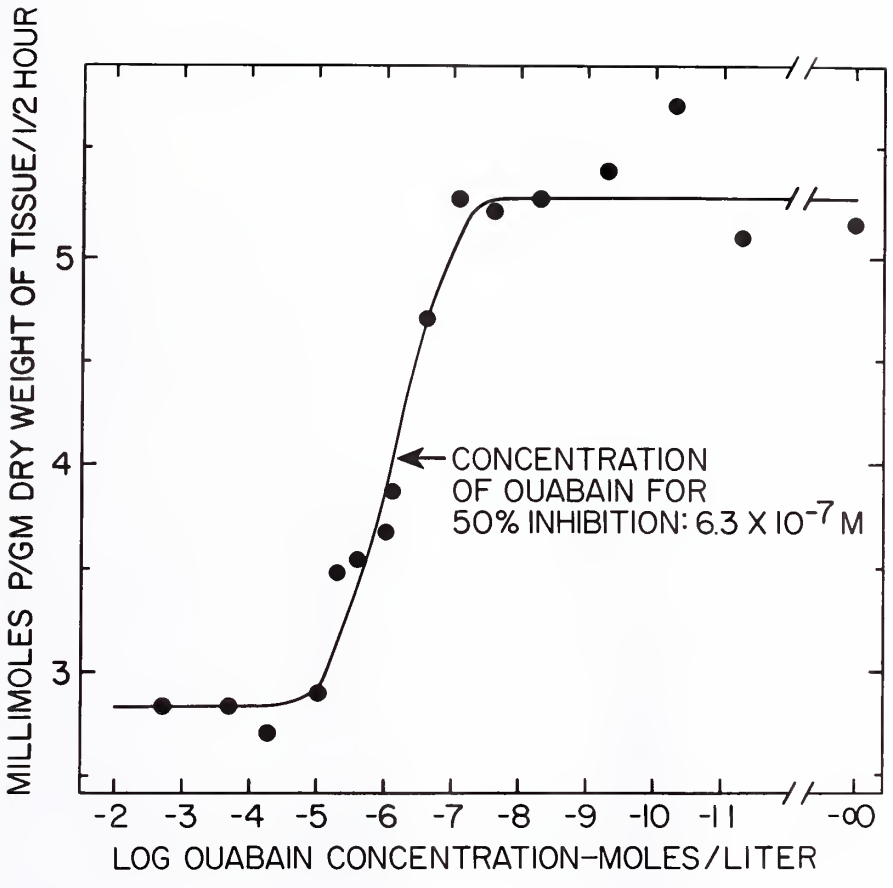

MILLIMOLES P/GM DRY WEIGHT OF TISSUE/1/2 HOUR
5
4
3
CONCENTRATION
OF OUABAIN FOR
50% INHIBITION: 6.3 X 10⁻⁷ M
-2 -3 -4 -5 -6 -7 -8 -9 -10 -11 -∞
LOG OUABAIN CONCENTRATION–MOLES/LITER

Figure 5

Enzymatic activity of pig rod outer segments as a function of pH. Medium contained 3 mM magnesium, 3 mM ATP, 20 mM K^+, 60 mM Na^+, 1 mM cysteine, and 0.1 mM EDTA. Buffers were 50 mM Tris and 50 mM imidazole brought to appropriate pH with HCl. Incubated 30 minutes at 37°C.

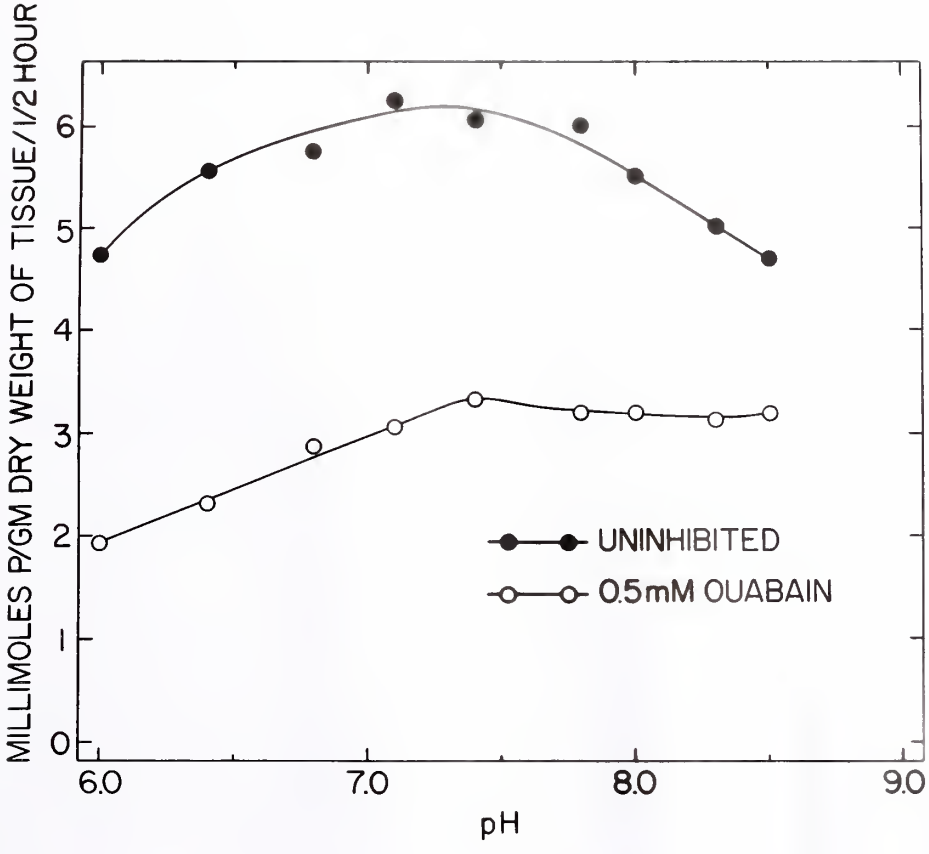

MILLIMOLES P/GM DRY WEIGHT OF TISSUE/1/2 HOUR
0
1
2
3
4
5
6
6.0
7.0
8.0
9.0
pH
UNINHIBITED
0.5mM OUABAIN

TABLE I

Specificity of Substrates

Substrate	millimoles P/gm dry weight/30 minutes		
	1 Uninhibited	2 0.5 mM ouabain	1-2 Ouabain-sensitive component
ATP	5.13 ± 0.39*	2.36 ± 0.17	2.77 ± 0.30
ADP	1.88 ± 0.14	1.23 ± 0.06	0.65 ± 0.08
AMP	0.86 ± 0.03	0.84 ± 0.09	0.02 ± 0.05
GTP	1.56 ± 0.10	1.16 ± 0.12	0.40 ± 0.11
CTP	2.02 ± 0.12	0.66 ± 0.07	1.36 ± 0.14
ITP	1.69 ± 0.10	1.11 ± 0.13	0.58 ± 0.10
UTP	1.19 ± 0.06	1.00 ± 0.17	0.19 ± 0.08
glucose-6 phosphate	0.25 ± 0.05	0.25 ± 0.05	0.00 ± 0.08
sodium beta-glycerol phosphate	0.19 ± 0.03	0.18 ± 0.03	0.01 ± 0.04

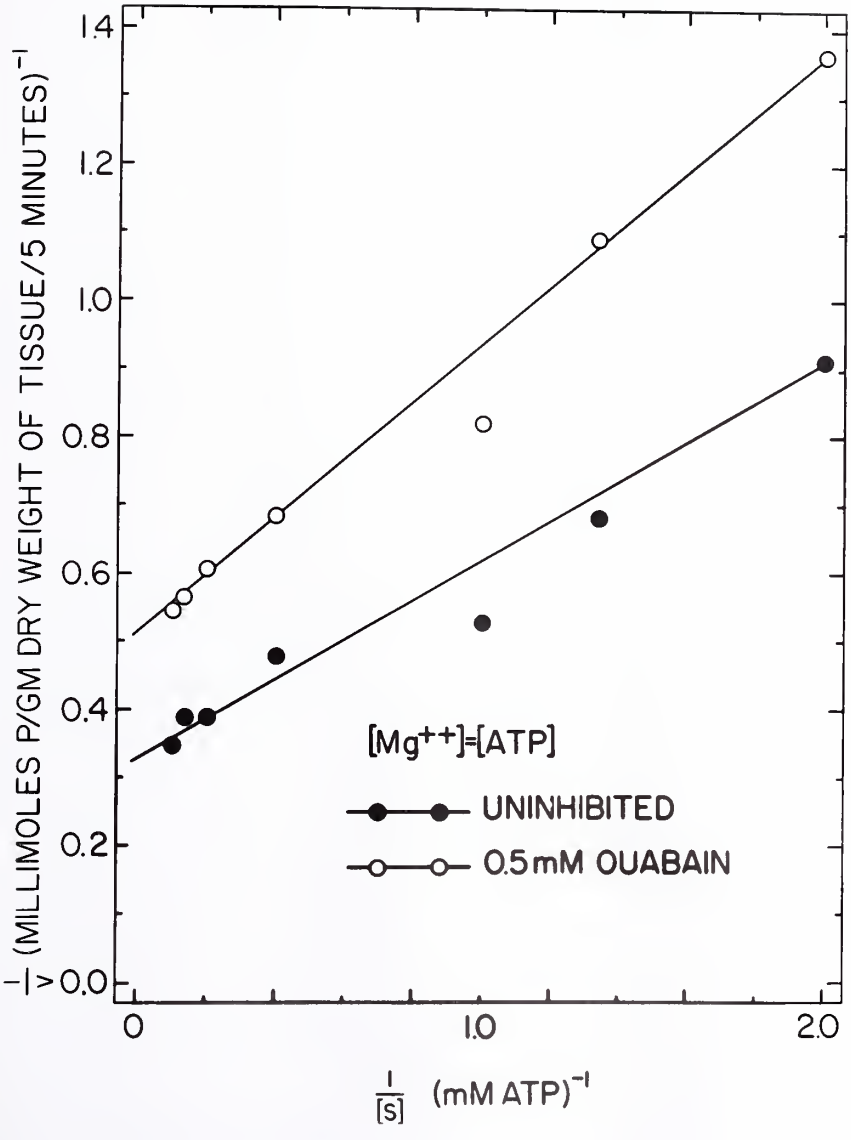
*Each figure is the average of four to six determinations ± the standard error of the mean. Incubation medium contained 3 mM substrate brought to pH 7.2 with Tris, 3 mM Mg^{++}, 20 mM K^{+}, 60 mM Na^{+}, 0.1 mM EDTA, 1 mM cysteine, and 50 mM Tris-HCl buffer, pH 7.2. Incubation for 30 minutes at 37°C.

component of the enzyme activity. Adenosine monophosphate was hydrolyzed scarcely at all, and as with ADP what hydrolysis did occur was almost entirely insensitive to ouabain. Because of the incubation time used in these experiments (30 minutes), the figures for ATP are not maximal rates. The substrate specificity may therefore be somewhat more pronounced than the results in Table I indicate.

Rate of enzyme action in light and darkness: The rate of ATP hydrolysis was determined for varying concentrations of ATP and Mg^{++}. Incubations were carried out in a 37°C. water bath, both in total darkness and in light from a 100-watt tungsten bulb placed approximately 1 foot from the incubation tubes. The bleaching light did not change the temperature of the bath. Figure 6 is the Lineweaver-Burke plot of the results of one experiment. Because there is reason to believe that these preparations contained more than one ATPase, the absolute values of the constants are of secondary interest; the principal point of these experiments is that no differences were observed between samples incubated in the dark and those in bright light. In four determinations in light and darkness, the V_{max} varied from 2.85 to 3.13 millimoles of phosphate released per gram dry weight of tissue per 5 minutes incubation. The K_m was 0.70-0.80 mM of ATP,

Figure 6

An example of a Lineweaver-Burke analysis of pig rod outer segment enzymatic activity as a function of ATP concentration. Magnexium and ATP concentrations were equal; other components as described under Figure 4. Incubated 5 minutes at 37°C. Same results were obtained in light and darkness.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1.0
2.0
1/v (MILLIMOLES P/GM DRY WEIGHT OF TISSUE/5 MINUTES)⁻¹
1/[s] (mM ATP)⁻¹
[Mg++]=[ATP]
UNINHIBITED
0.5mM OUABAIN

and the K_i for ouabain, 0.80-1.76 mM. Ouabain inhibition was noncompetitive.

Comparison of ATPase activity from rod outer segments with ATPase activity in the remainder of the retina and in pig liver mitochondria: Incubations were carried out in dark and in light, using both lyophilized outer segments and retinal remainders. Furthermore, several substances were tested for their effects on activation or inhibition of the enzymes from the two sources. Table II shows that light and dark had no effect, with or without 100 mM hydroxylamine. 3 mM calcium produced an inhibition, presumably by competing with magnesium; 2 mM PCMB inhibited activity, presumably because ATPase activity is sulfhydryl-dependent; 0.1 mM DNP had no effect.

Since it was probable that these preparations of rod outer limbs contained some mitochondrial contamination, their activity was compared with that of a lyophilized preparation of pig liver mitochondria. Table III shows that (i) mitochondria contain less ATPase activity on a dry weight basis than do rod outer segments; and (ii) the mitochondrial ATPase activity is activated by magnesium, but there is no further activation by sodium and potassium, nor is there inhibition by ouabain. Similar effects of ions and ouabain were reported for rat brain mitochondria by Van Groningen and Slater (1963). Although it is

TABLE II

Comparison of ATPase Activity in Pig Outer Segments and Inner Retinal Layers

Sample	millimoles P/gm dry weight/5 minutes	
	Outer Segments	Inner Retina
Dark control	2.01 ± 0.06*	1.94 ± 0.20
Light control	2.04 ± 0.03	1.95 ± 0.15
100 mM hydroxylamine, dark	1.90 ± 0.06	1.76 ± 0.14
100 mM hydroxylamine, light	1.96 ± 0.04	1.79 ± 0.14
3 mM Ca^{++}	0.72 ± 0.08	0.71 ± 0.09
2 mM PCMB	0.57 ± 0.03	0.64 ± 0.08
0.5 mM ouabain	0.91 ± 0.04	0.99 ± 0.09
0.1 mM DNP	1.94 ± 0.06	2.06 ± 0.22

*Figures are averages of six determinations, with standard error of the mean. Tubes contained 20 mM K^{+}, 60 mM Na^{+}, 3 mM Mg^{++}, 3 mM ATP, 0.1 mM EDTA, 1 mM cysteine, 50 mM Tris-50 mM imidazole buffer, pH 6.8. Approximately 0.7 mg tissue per milliliter. Incubated 5 minutes at 37 C.

Table III

Comparison of ATPase Activity in Pig Outer Segments and Pig Liver Mitochondria

Source	millimoles P/gm dry weight/30 minutes				
	No Mg^{++}	Mg^{++}	Mg^{++}, Na^{+}, K^{+}	Mg^{++}, ouabain	Mg^{++}, Na^{+}, K^{+}, 0.5 mM ouabain
Outer segments	0.31 ± 0.01*	2.34 ± 0.04	5.54 ± 0.08	2.41 ± 0.10	2.42 ± 0.08
Liver mitochondria	0.35 ± 0.01	1.74 ± 0.03	1.73 ± 0.06	----------	1.57 ± 0.10

*Figures are averages of four determinations, with standard errors of the mean. All tubes contained 0.1 mM EDTA, 1 mM cysteine, 50 mM Tris-HCl buffer, pH 7.2, 3 mM ATP, and approximately 7 mg (dry weight) tissue per milliliter. Incubation was for 30 minutes at 37°C. Concentrations of cations were: Mg^{++} 3 mM, K^{+} 20 mM, Na^{+} 60 mM. Ouabain, when present, was employed in a concentration of 0.5 mM.

probable that some of the magnesium-dependent ATPase activity that was measured in preparations of outer limbs comes from mitochondria, the preponderance of the activity, including all of the sodium-potassium dependent activity, is not mitochondrial in origin. Since the sodium-potassium dependent activity constitutes about 50% of the total, it is apparent that not more, and indeed probably considerably less, than 50% of the total "outer segment" ATPase activity measured in these experiments arises in mitochondria.

Effects of extracting agents: 2% digitonin and 4% CTAC, two agents which are known to extract rhodopsin from the rod outer segments (Dartnall, 1957), were employed in order to compare the extraction of ATPase activity with that of the visual pigment. CTAC is an efficient solubilizer of rhodopsin, but negligible ATPase activity was detected in the rhodopsin-containing supernatant, the washed sediment, or in uncentrifuged suspensions containing CTAC. Distilled water controls incubated under the same conditions showed considerable activity (Table IV).

Digitonin, on the other hand, extracts ATPase activity as well as rhodopsin (Table V), an observation which has also been made by McConnell and Scarpelli (1963) and by Bonting et al. (1964). In agreement with these latter

TABLE IV

Distribution of Rhodopsin and ATPase Activity after Extraction with 4% CTAC

Sample	0.5 mM Ouabain	millimoles P/gm dry weight/30 min.	Rhodopsin** ΔE_{500} x (V/W)
Control: Rod powder suspended in H_2O	0	3.02 ± 0.41*	----
	+	2.14 ± 0.20	
Control: Rod powder suspended in 4% CTAC; supernatant and sediment not separated	0	0,090 ± 0.004	
	+	0.092 ± 0.003	----
Supernatant, 90 minute extraction	0	0.092 ± 0.002	21.9
	+	0.099 ± 0.007	
Sediment, 90 minute extraction	0	0.072 ± 0.003	0.0
	+	0.074 ± 0.005	

*Figures are averages of four determinations with standard errors of the mean. Rhodopsin concentration is expressed as ΔE_{500} x (V/W), where ΔE_{500} is the change in extinction at 500 mμ on bleaching, V is the volume of the sample in ml, and W is the weight of the tissue in gm. Incubation medium for enzyme assays contained 3 mM ATP brought to pH 7.2 with Tris, 20 mM K^+, 60 mM Na^+, 3 mM Mg^{++}, 0.1 mM EDTA, 1 mM cysteine, and 50 mM Tris-HCl buffer, pH 7.2. Incubation for 30 minutes at 37°C.

**It is difficult to estimate with accuracy the amount of rhodopsin in a very turbid suspension. The amount of rhod-

opsin recovered in the digitonin extract is a reasonable estimate of the amount originally present, as there is no evidence that rhodopsin is destroyed by digitonin. However, in these experiments there is some variation in the amount of rhodopsin recovered because uniformly dark-adapted eyes were not available. The amount of rhodopsin in CTAC extracts and its subsequent stability are evidence that CTAC does not destroy rhodopsin either.

TABLE V

Distribution of Rhodopsin and ATPase Activity after Extraction with 2% Digitonin

Sample	0.5 mM Ouabain	millimoles P/gm dry weight/30 min.	Rhodopsin** ΔE_{500} x (V/W)
Control: Rod Powder suspended in H_2O	0	3.20 ± 0.14*	----
	+	1.76 ± 0.09	
Control: Rod powder suspended in 2% digitonin; supernatant and sediment not separated	0	0.79 ± 0.02	----
	+	0.53 ± 0.05	
Supernatant, 90 minute extraction	0	0.67 ± 0.01	16.6
	+	0.52 ± 0.04	
Sediment, 90 minute extraction	0	0.34 ± 0.02	0.0
	+	0.22 ± 0.02	

*Figures are averages of four determinations, with standard errors of the mean. Rhodopsin concentration is expressed as ΔE_{500} x (V/W). Meaning of the terms in this formula is explained in first footnote under Table IV. Incubation medium was the same as that used in experiments in Table IV.

**See second footnote under Table IV.

authors, however, the present results show that digitonin inhibits the hydrolysis of ATP. The supernatant from such an extraction contained only about 20% of the ATPase activity originally present. Approximately 30% of the original ouabain-insensitive component was present, but only about 10% of the ouabain-sensitive component. All of the rhodopsin recovered was in the supernatant.

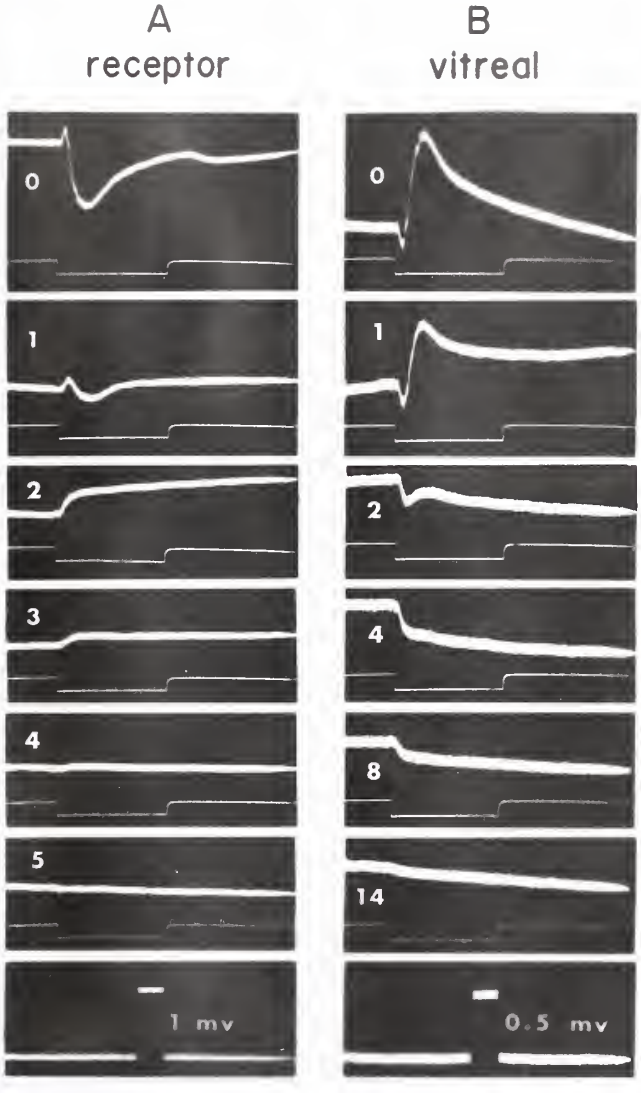
The sediment from such a digitonin extraction contained 8% of the total ATPase activity originally present. This consisted of approximately 9% of the original ouabain-insensitive component and about 7% of the ouabain-sensitive fraction. No rhodopsin could be detected in the sediment.

II. Electrophysiological Experiments on Isolated Frog Retinas

Effects of ouabain on isolated retinas: When a test solution containing a relatively high concentration of ouabain (10^{-3}M) was applied to the receptor surface of the isolated retina, the electrical response of the retina to test flashes of light was rapidly abolished (Figure 7A). Within 30 seconds, the b-wave began to diminish, and 2-3 minutes after the application of ouabain the b-wave had entirely disappeared, leaving only the isolated P III component. This at first appeared somewhat

Figure 7

Effect on isolated frog retina of Ringer with 10^{-3}M ouabain. (A) Receptor surface oriented up; solution dripped directly onto receptors; (B) Vitreous surface up; solution dripped onto vitreous surface. Upper traces record electrical responses of retina, lower traces record duration of light stimulus. Bottom record is calibration pulse. Numbers indicate minutes after application of test solution. Experimental conditions described in text.



10^{-3}M. ouabain

larger in amplitude than the original a-wave, possibly because of the removal of the b-wave, which has opposite polarity and hence tends to cancel P III. However, the isolated P III soon began to diminish, and after five minutes no further electrical response could be obtained. This effect was irreversible, for if the retina were now bathed in fresh Ringer every five minutes, no response could be observed when test flashes were given at five minute intervals for 30 minutes.

When 10^{-3}M ouabain was applied to the inner (vitreous) surface of the retina, the ERG decayed much more slowly (Figure 7B). The b-wave disappeared after 4-5 minutes, and the P III component could be detected for 12-15 minutes after the application of the glycoside.

In neither case did the isolated P III component return to the baseline within 10 seconds after cessation of the stimulus. This persistence of the isolated P III has been called "remnant negativity" (Granit, 1962a). It probably reflects receptor potentials from rod cells virtually alone, since Brown and Watanabe (1962b) find that P III in cones cuts off sharply after cessation of illumination, while the receptor potential in rods is considerably prolonged. The d-wave is also held to be predominantly a cone response (Granit, 1962a; Brown and Watanabe, 1962b) and in the present experiments, utilizing a relatively low-intensity stimulus flash which should

primarily have stimulated the rods, the d-wave was never prominent. (See also previous discussion, pp. 41-42.)

When lower concentrations of ouabain were applied to isolated retinas, the effects were slower and were qualitatively slightly different. Following the application of 10^{-4}M ouabain to the receptor surface (Figure 8A), four minutes were required to abolish the b-wave, and 6-8 minutes elapsed before P III also disappeared. When 10^{-4}M ouabain was applied to the vitreous surface (Figure 8B) the b-wave diminished much more slowly, and was at first accompanied by an apparent increase in the amplitude of the a-wave (the leading edge of P III). After 30 minutes, the b-wave was entirely absent and P III began to decay, but it was still evident 40 minutes after the initial application of ouabain.

When yet lower concentrations of ouabain were applied to frog retinas, the b-wave appeared to decrease more rapidly than the a-wave, but an isolated P III was not observed. In the late stages of these experiments, both a- and b-waves were markedly reduced, and both eventually disappeared together.

The smallest concentration of ouabain which had a consistent effect on the ERG was 10^{-6}M (Figure 9). The effect at this concentration appeared to be slightly reversible. Thus, if at the end of an 80-90 minute incu-

Figure 8

Effect on electrical activity of isolated frog retina of Ringer with 10^{-4}M ouabain. (A) Solution applied to upward-oriented receptor surface; (B) solution applied to upward-oriented vitreous surface. Other details as described under Figure 7.

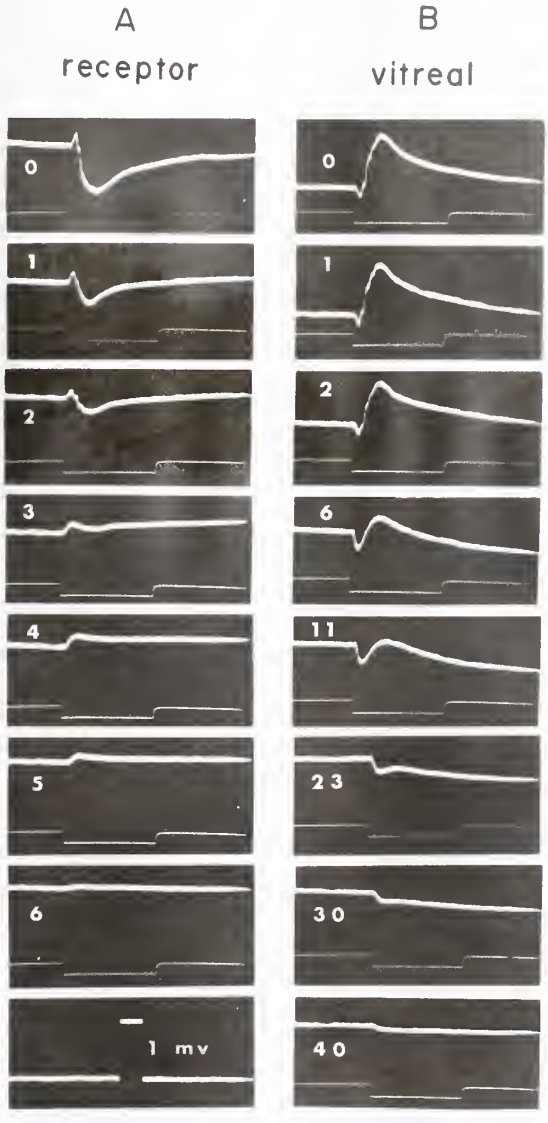
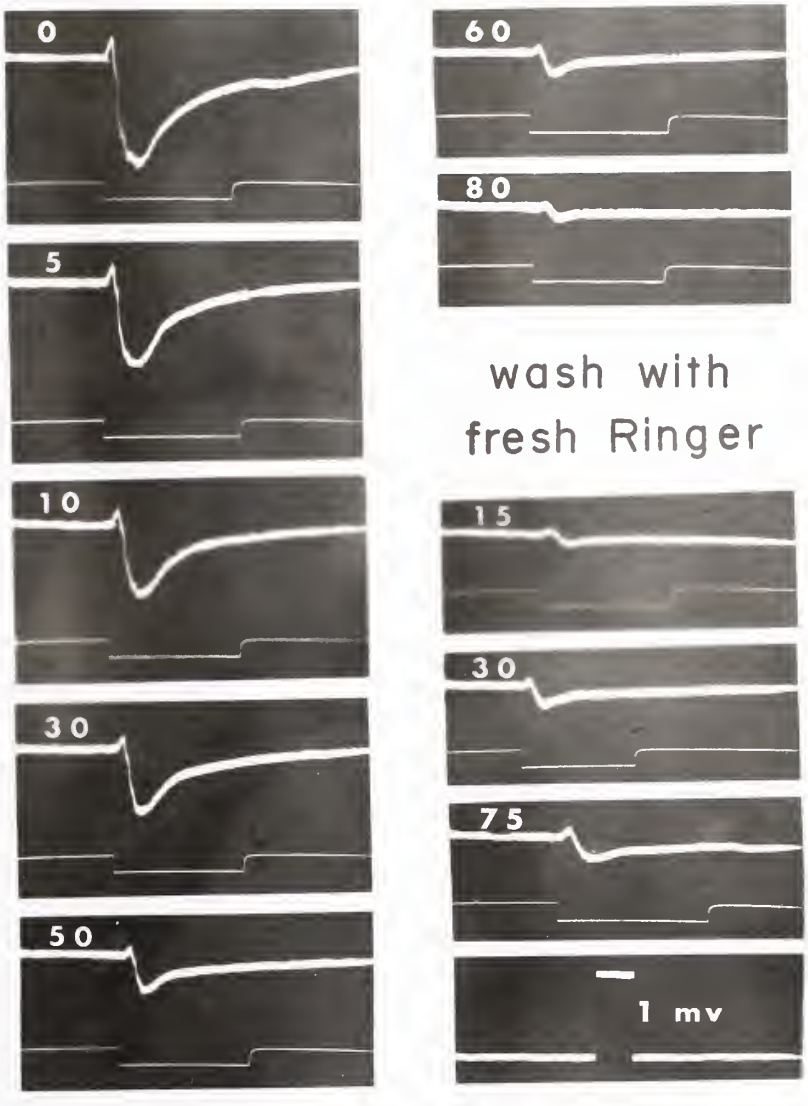

A
receptor
B
vitreal
0
1
2
3
4
5
6
1 mv
0
1
2
6
11
23
30
40
10^{-4}M. ouabain

Figure 9

Effect on electrical activity of isolated frog retina of Ringer with 10^{-6}M ouabain applied to receptor surface. After 80 minutes the test chamber and the retina were washed twice with fresh Ringer without glycoside. Fresh Ringer was then dripped on the retina every five minutes for the duration of the experiment. Details as described in text, and under Figure 7.



10^{-6}M. ouabain

bation with 10^{-6}M ouabain, the retina was washed at five minute intervals with fresh Ringer without glycoside, a small increase in the amplitude of the response (both a- and b-waves) could be seen over a 75 minute period. The final responses, however, were never more than 25-30% of the original amplitude (Figure 9). In the presence of 10^{-6}M ouabain a response to test flashes could be evoked for more than 100 minutes.

With 10^{-7}M ouabain, no change in the response occurred over a 90 minute period. This may, however, suggest a small effect of ouabain, because in several control experiments in which Ringer without glycosides or ethanol was used as the test solution, the a- and b-waves increased slightly in amplitude, the b-wave peak became much sharper, and the d-wave became slightly more prominent.

Effects of other glycosides on electrical activity: In order to demonstrate that these effects of ouabain on the electrical activity of the retina are caused by inhibition of the sodium transport system rather than through some other property of the glycoside, another approach was taken. The experiments were suggested by the work of Glynn and his associates (Glynn, 1957b; Dunham and Glynn, 1961), who studied the effects of slight modifications of the glycoside molecule on active sodium-potassium

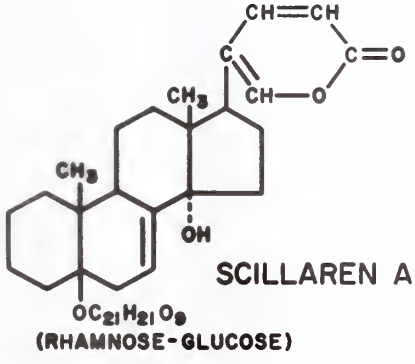
transport and on the sodium-potassium activated ATPase system in human erythrocyte membranes. They showed that hydrogenation of the lactone ring, or placing the lactone ring at carbon-17 in the alpha instead of the beta orientation markedly reduced the effectiveness of the glycoside molecule both as an inhibitor of active sodium-potassium transport and of the related ATPase activity. That such slight alterations can produce significant effects strongly suggests that these particular configurational changes have impaired the fit of the glycoside molecule into a specific inhibitory site on a particular enzyme.

Experiments were performed with the glycosides scillaren A and cymarin, which have been found to inhibit both ATPase activity and the active transport system for sodium and potassium in human red blood cells, and with hexahydroscillaren A, in which the lactone ring of scillaren A is hydrogenated, and 17-alpha cymarin, which has the lactone ring at C-17 in the alpha and not the beta orientation. These latter two glycosides are considerably less effective inhibitors of transport ATPase activity and of the sodium-potassium pump (Glynn, 1957b; Dunham and Glynn, 1961; Glynn, 1964). The molecular structures are shown in Figure 10.

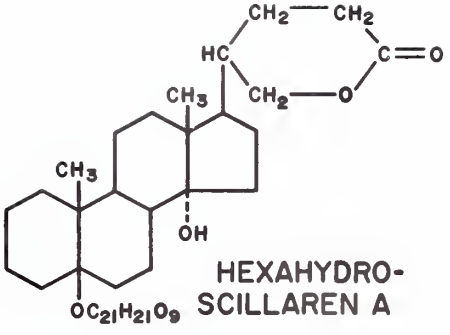
Figure 11A shows oscillograms from a control experiment in which the test solution was ringer containing 4% ethanol. The changes produced by 4% ethanol are simi-

Figure 10

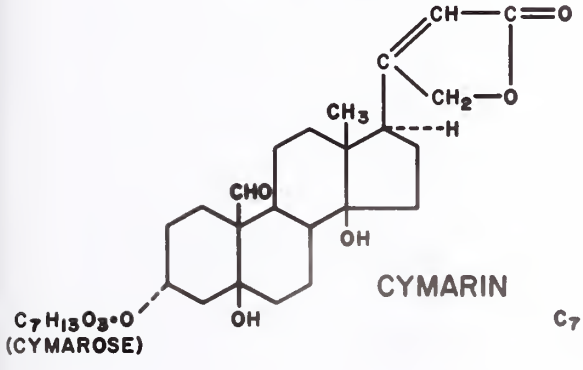
Structural formulas of glycosides used in these experiments. Scillaren A and cymarin are more effective inhibitors of Na^+-K^+ activated ATPase and active sodium-potassium transport in human erythrocyte membranes than are hexahydroscillaren A and 17-alpha cymarin.



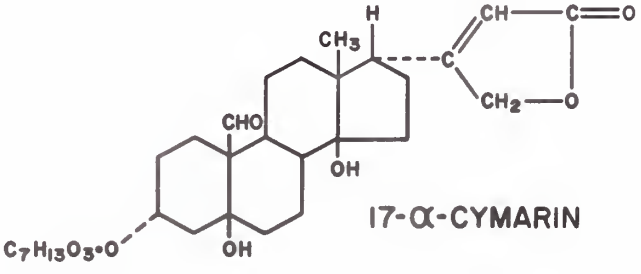
SCILLAREN A



HEXAHYDRO-
SCILLAREN A



CYMARIN



17-α-CYMARIN

lar to those described by Bernhard and Skoglund (1941). Very soon after the application of the ethanolic solution, there is a marked increase in the amplitude of the b-wave and a decrease in its rate of decay. The a-wave appears to diminish. The amplitude of the b-wave gradually decreases, and in several experiments it had become somewhat smaller than its initial size by 30-40 minutes.

Figure 11B shows the effect of 10^{-4}M scillaren A in 4% ethanol-Ringer. Within eight minutes the retina was no longer responsive. Initially, the a-wave decreased in amplitude, while the b-wave changed its form, but did not decline as rapidly. Indeed, in some experiments, it temporarily increased (Figure 11B). On the basis of control experiments without glycoside, this would appear to be a reflection of the effects of ethanol. After 4-5 minutes, the b-wave had disappeared, leaving the isolated P III component, which itself disappeared within 7-8 minutes of the beginning of the experiment. The effect of cymarin was similar.

When tested at concentrations of 10^{-4}M, hexahydroscillaren A and 17-alpha cymarin were also effective in abolishing the response to light, but their action was significantly less rapid than was that of scillaren A and cymarin. The effect of 10^{-4}M hexahydroscillaren A applied to the receptor surface is shown in Figure 11C; the results

with 17-alpha cymarin were similar, but required a somewhat shorter time. Shortly after application of these glycosides, the b-wave increased and the a-wave apparently decreased, once again most probably the effect of ethanol. This transitory enhancement of the b-wave was more prominent in experiments with hexahydroscillaren A and 17-alpha cymarin than with scillaren A and cymarin because the latter glycosides, being more effective inhibitors of the response, tend more rapidly to cancel the oppositely directed effects of 4% ethanol.

Figures 12 and 13 show the time courses of inhibition of the response to light when these glycosides were used as test substances at 10^{-4}M. For comparison, the time course of change in the ERG caused by 4% ethanol-Ringer is also shown. The ordinate is the size of the response --a- and b-waves together--expressed as a percentage of the control measured before applying the test solution. Although the a- and b-waves are probably the results of separate processes, which very likely occur at different sites in the retina, plotting their sum does not obscure the conclusion that slight changes in the molecular structure of glycosides can markedly alter their effectiveness as inhibitors of electrical activity of the retina.

From Figure 14 one can determine the relative effectiveness of the four glycosides as inhibitors of the electrical activity of the retina. The points and curve show the

Figure 11

(A) Left column: Effect on isolated frog retina of Ringer with 4% (v/v) ethanol applied to receptor surface; (B) Middle column: Effect on isolated frog retina of 4% ethanol-Ringer with 10^{-4}M scillaren A applied to receptor surface; (C) Right column: Effect on isolated frog retina of 4% ethanol-Ringer with 10^{-4}M hexahydroscillaren A applied to receptor surface. Further explanation of this figure as described under Figure 7. Experimental details described in text.

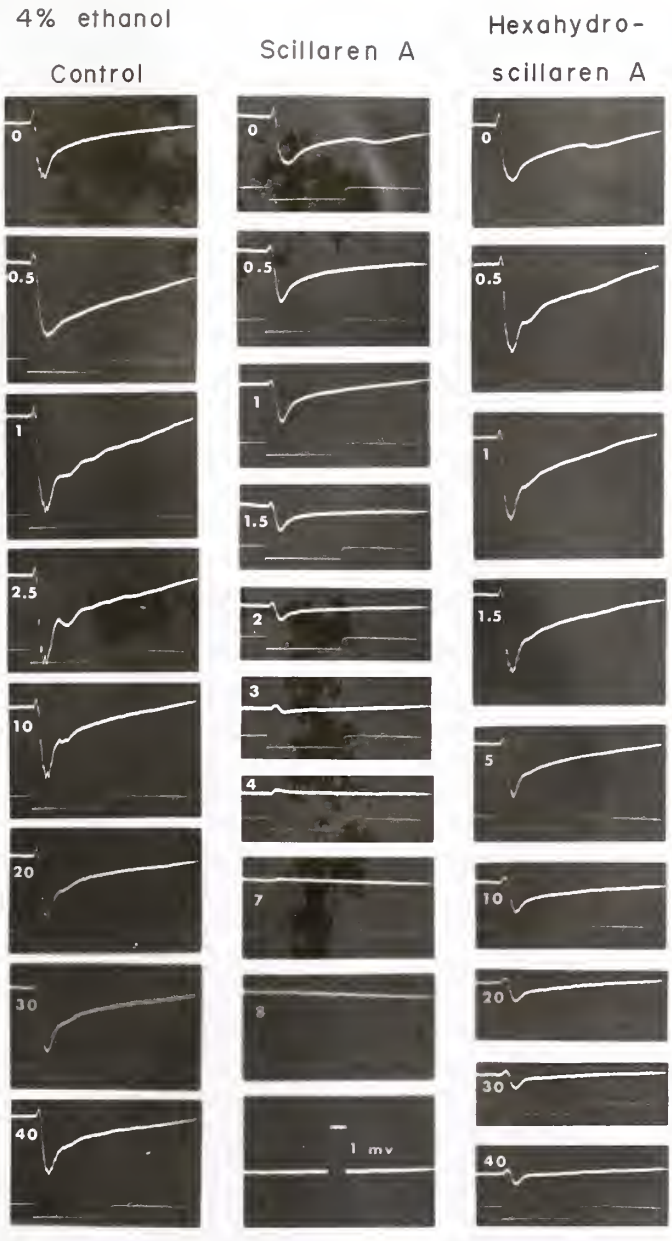

4% ethanol
Control
Scillaren A
Hexahydro-
scillaren A
0
0.5
1
2.5
10
20
30
40
0
0.5
1
1.5
2
3
4
7
8
1 mv
0
0.5
1
1.5
5
10
20
30
40

Figure 12

Time course of decay of ERG (a- and b-waves measured together) after application of 4% ethanol-Ringer (top curve), 4% ethanol-Ringer with 10^{-4}M hexahydroscillaren A (middle curve), and 4% ethanol-Ringer with 10^{-4}M scillaren A (bottom curve) to receptor surface of isolated frog retina. Standard error of at least four determinations shown for each point. Ordinate shows amplitude of electrical response, as percent of control ERG (at zero time), summing a- and b-waves together.

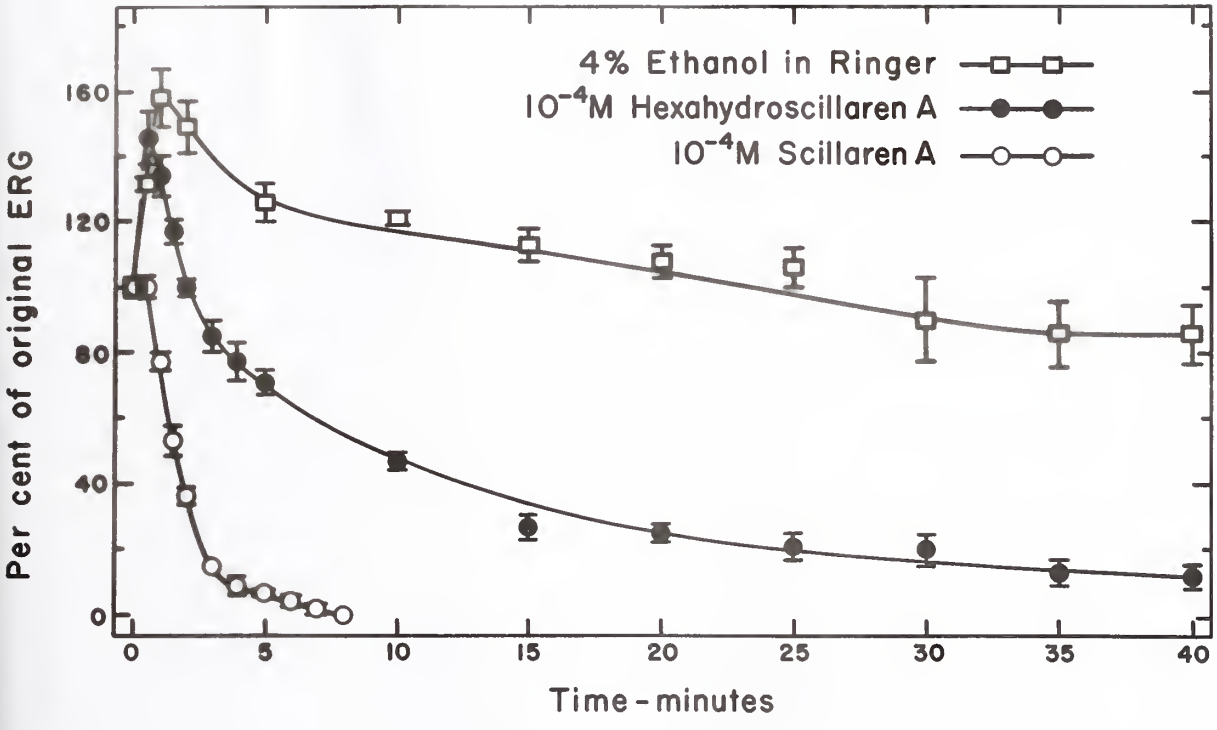
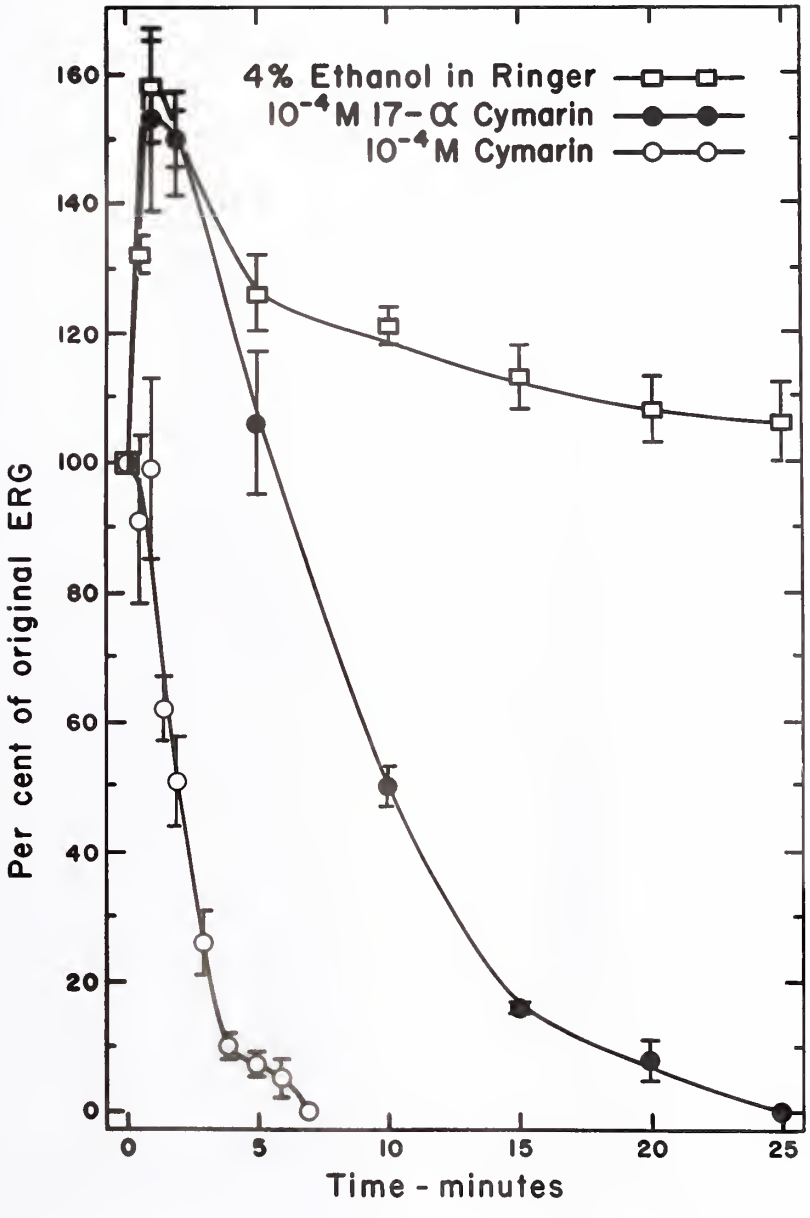

4% Ethanol in Ringer
10⁻⁴M Hexahydroscillaren A
10⁻⁴M Scillaren A
Per cent of original ERG
160
120
80
40
0
0
5
10
15
20
25
30
35
40
Time - minutes

Figure 13

Time course of decay of ERG (a- and b-waves together) after application of 4% ethanol-Ringer (top curve), 4% ethanol-Ringer with 10^{-4}M 17-alpha cymarin (middle curve), and 4% ethanol-Ringer with 10^{-4}M cymarin (bottom curve). Standard error of at least four determinations shown for each point. Further details as described under Figure 12.


4% Ethanol in Ringer
10⁻⁴M 17-α Cymarin
10⁻⁴M Cymarin
Per cent of original ERG
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
Time - minutes

reciprocal of the time required to abolish completely the ERG as a function of the logarithm of the concentration of ouabain. The relative inhibitory effectiveness of cymarin, scillaren A, 17-alpha cymarin, and hexahydroscillaren A are determined by finding on the curve the concentration of ouabain necessary to produce the same effect on the ERG as 10^{-4}M of each glycoside. Figure 14 is representative of a series of four curves which were plotted for the purpose of making this comparison. The other three curves plotted the logarithm of the ouabain concentration (on the abscissa) against reciprocal time to reduce the ERG (a- and b-waves summed together) to 75%, 50%, and 25% of its control amplitude. For each of the four glycosides, therefore, four equivalent concentrations of ouabain could be determined with electrical effects similar to 10^{-4}M of the glycoside. These four figures were averaged to give a mean value, which is shown in the first column of Table VI, $\pm$ the standard error of the mean. The second column compares the effectiveness of these four glycosides as inhibitors of the ERG in the isolated frog retina, relative to the most effective, scillaren A, as 100.

Effects of cardiac glycosides on the ATPase of frog outer segments: To strengthen the conclusion that the abolition of electrical responses was caused by inhibi-

Figure 14

Rate of decay of ERG (a- and b-waves together) as a function of glycoside concentration. Abscissa: logarithm of ouabain concentration. Ordinate: reciprocal time for complete abolition of ERG; units are $(\text{minutes})^{-1}$. This function is proportional to rate, and has the same units. Rate of effect for 10^{-4}M scillaren A, cymarin, hexahydro-scillaren A, and 17-alpha cymarin are plotted on this curve, enabling one to compare quantitatively their relative effectiveness. All points are the means of at least four determinations. Similar curves (not shown) were plotted using as ordinate reciprocal time to reduce the ERG to 75%, 50%, and 25% of its control value. Data in Table VI was calculated from these four curves. (See text, pp. 55-56.)

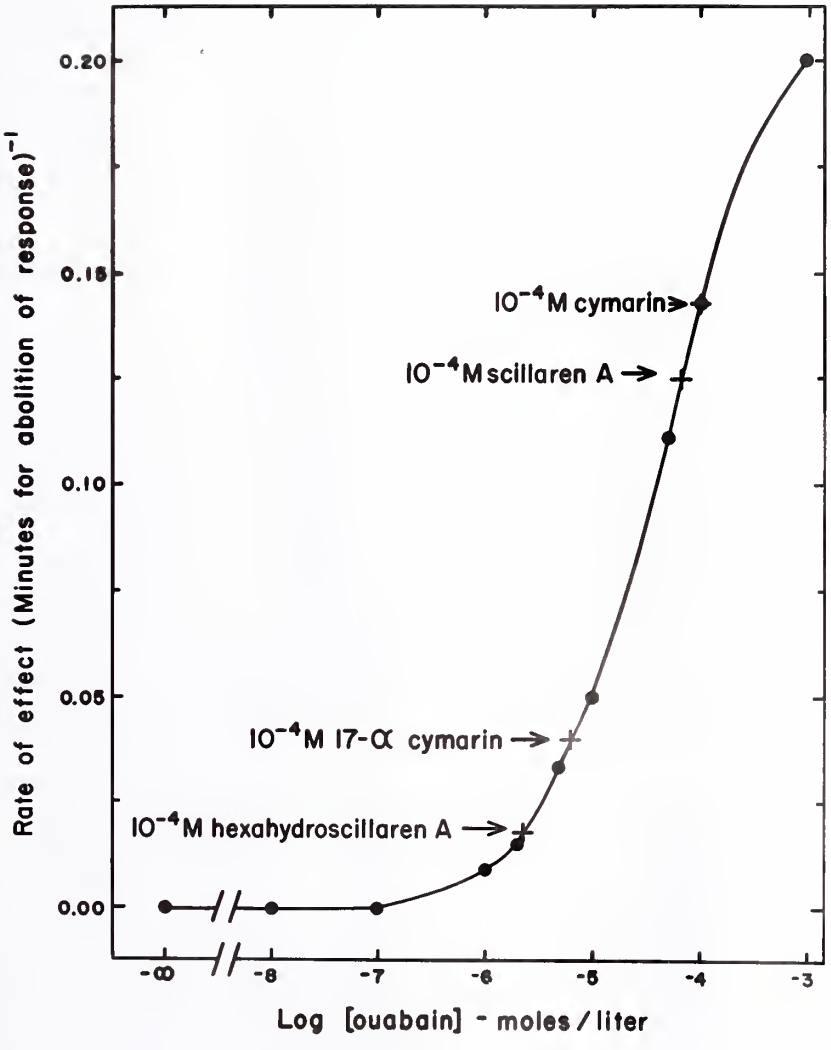

0.20
0.15
0.10
0.05
0.00
Rate of effect (Minutes for abolition of response)$^{-1}$
10^{-4}M cymarin
10^{-4}M scillaren A
10^{-4}M 17-α cymarin
10^{-4}M hexahydroscillaren A
-∞
-8
-7
-6
-5
-4
-3
Log [ouabain] - moles / liter

Table VI

Relative Effectiveness of Four Glycosides in Abolishing the ERG of Isolated Frog Retinas and as Inhibitors of Na^+-K^+ ATPase of Frog Retinal Outer Segments

Glycosides	concentration of ouabain (M) required to produce same effect as 10^{-4}M glycoside[1]	relative inhibitory effectiveness on electrical response[2]	concentration (M) required to produce half-maximal inhibition of ATPase	relative inhibitory effectiveness on ATPase
Scillaren A	$(9.2 \pm 0.8) \times 10^{-5}$	100	2.0×10^{-8}	100
Cymarin	$(6.8 \pm 1.4) \times 10^{-5}$	74	2.0×10^{-8}	100
Hexahydro-scillaren A	$(7.1 \pm 2.0) \times 10^{-6}$	7.7	5.0×10^{-6}	0.4
17-alpha cymarin	$(6.9 \pm 0.0) \times 10^{-6}$	7.5	2.5×10^{-6}	0.8

[1] See Figure 14 and explanation in text.

[2] Based on scillaren A as 100.

tion of the sodium pump, the same glycosides were tested on the sodium-potassium activated ATPase of frog retinal outer limbs.

Under the present assay conditions, maximum total ATPase activity of outer segments was approximately 2.5 millimoles phosphate released per gram dry weight of tissue per 30 minutes at 37°C., or about half the rate observed for pig outer segments. About one-third the ATPase activity of frog outer segments is Na^+-K^+ dependent and glycoside inhibited. Bonting et al. (1964) found that about two-thirds of the ATPase of frog outer segments was Na^+-K^+ dependent, although their figures for total activity are comparable to mine when expressed in the same units. The reason for this discrepancy is not clear.

The inhibitory effect of ouabain on Na^+-K^+ dependent ATPase activity in frog retinal outer segments is shown in Figure 15. The concentration necessary for half-maximal inhibition is 1×10^{-7}M. The effects of the other four glycosides tested are shown in Figures 16 and 17. For each glycoside studied, enzyme activity was determined as a function of the logarithm of the glycoside concentration. To determine the expected ATPase activity when the glycoside inhibition was maximal, tubes were incubated without potassium or glycoside. For unknown reasons, there was considerable scatter of individual points in determinations of ATPase inhibition of glycosides other than ouabain,

Figure 15

ATPase activity of frog retinal outer segments as a function of ouabain concentration. Medium contained 1.4 mg dry weight of tissue/ml, 3 mM Mg^{++}, 3 mM ATP, 60 mM Na^{+}, 20 mM K^{+}, 1 mM cysteine, 0.1 mM EDTA, 67 mM Tris buffered to pH 7.2 with HCl. All cations present as chlorides. Arrow on curve indicates half-maximal enzyme activity. Other conditions of assay described in text.

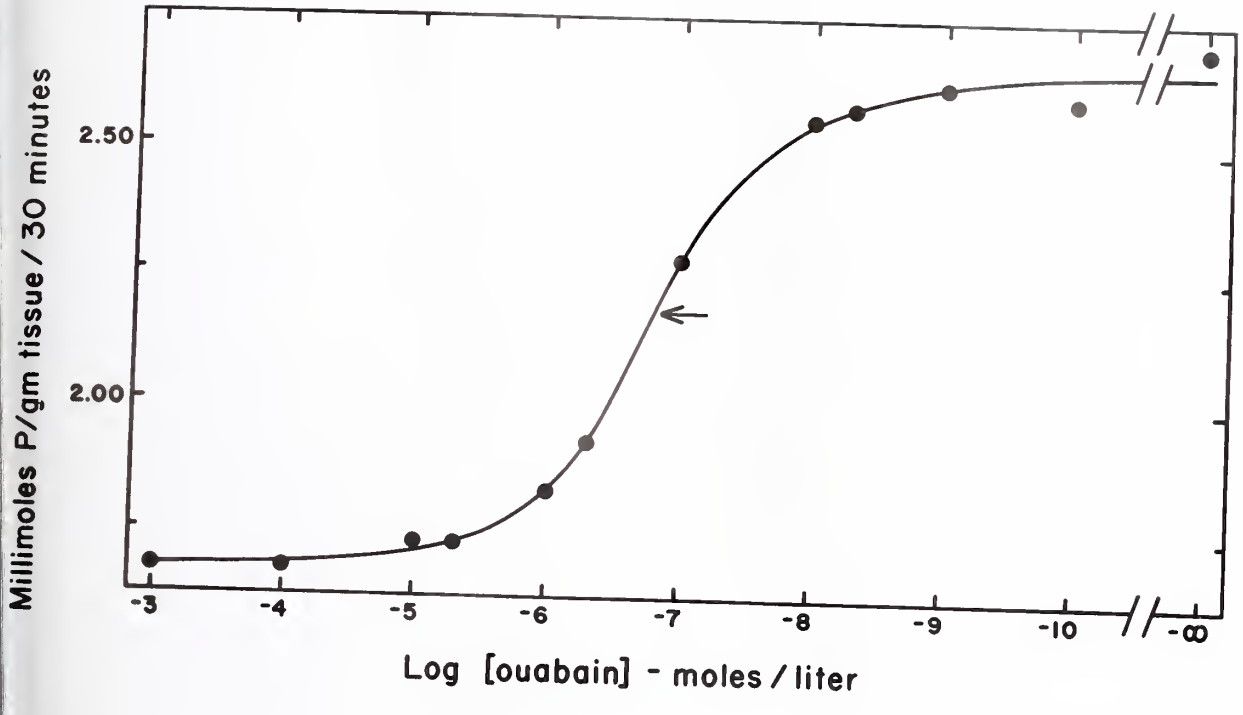

Millimoles P/gm tissue / 30 minutes
2.50
2.00
-3
-4
-5
-6
-7
-8
-9
-10
-∞
Log [ouabain] - moles / liter

Figure 16

ATPase activity of frog retinal outer segments as a function of glycoside concentration. Upper curve, hexahydroscillaren A. Lower curve, scillaren A. Each point is mean ± standard error of the mean of four determinations. (Standard errors shown by vertical bars.) Because of random daily variations of total enzyme activity, average results are plotted as percent of maximum enzyme activity, rather than as specific units, on the ordinate. 100% activity is obtained in tubes containing full assay medium and no inhibitor. Theoretical maximal glycoside inhibition is obtained in tubes containing sodium, but no potassium or glycoside. This is indicated by the point marked with the symbol "X" at the lower left of this figure and Figure 17. The appropriate standard error is also indicated for this point, which is used to calculate the glycoside concentration for half-maximal inhibition. The dashed lines in this figure and Figure 17 indicate theoretical extensions of the inhibition curves to include this point for maximal inhibition. Arrows indicate half-maximal inhibition. All assay tubes contained 0.4% (v/v) ethanol. Other contents described under Figure 15. Assay conditions described in text.

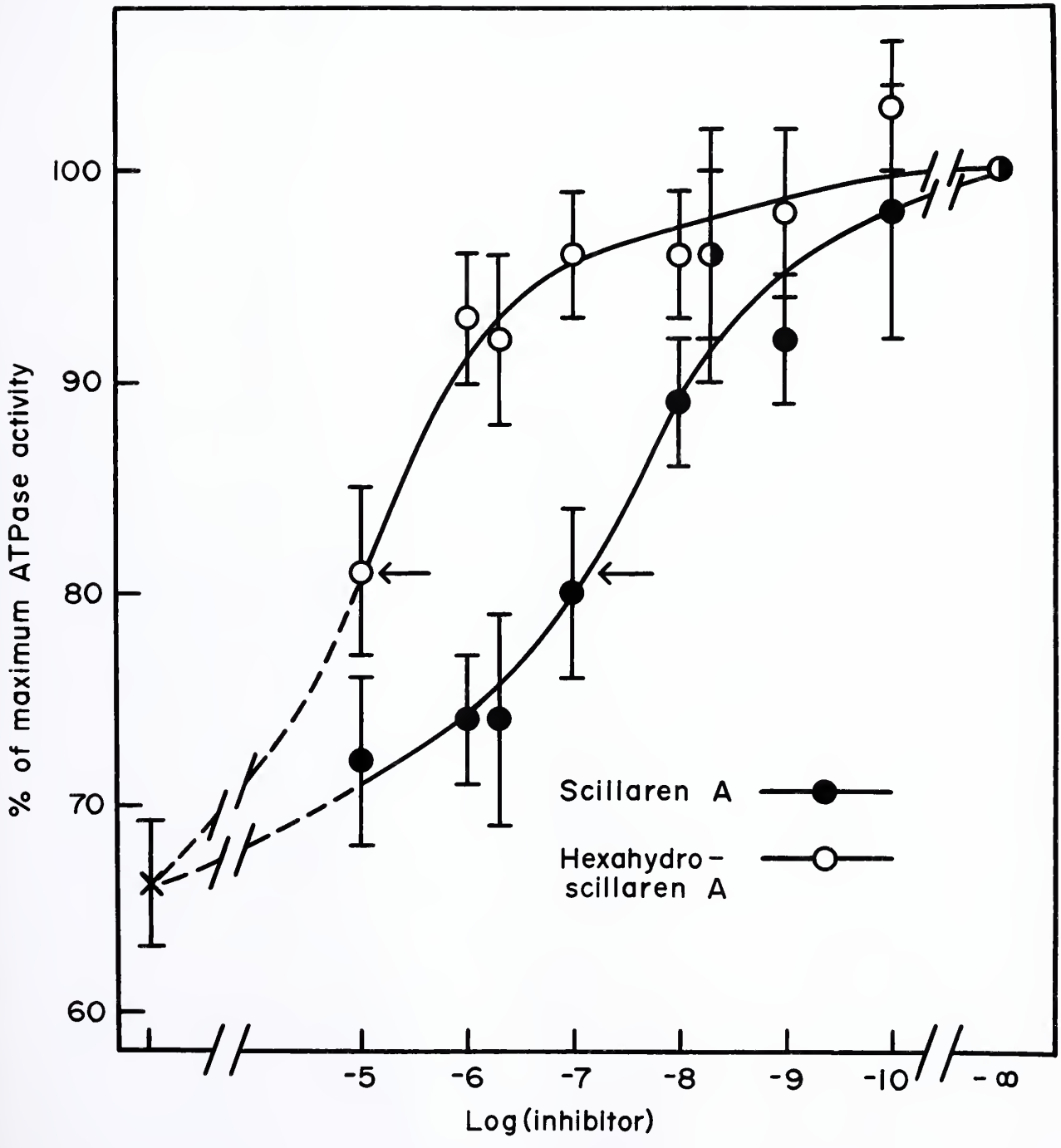
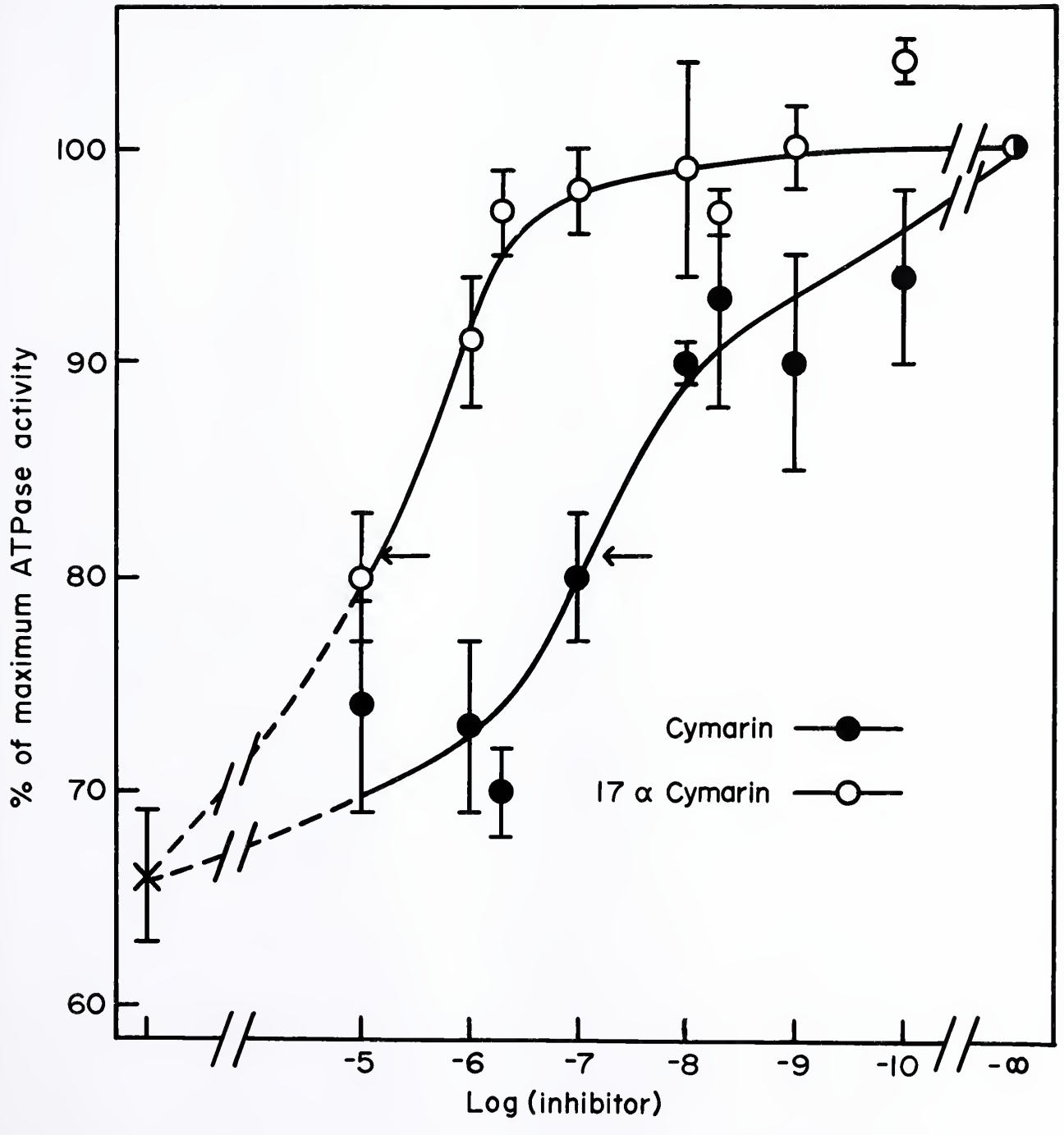

100
90
80
70
60
% of maximum ATPase activity
-5
-6
-7
-8
-9
-10
-∞
Log (inhibitor)
Scillaren A
Hexahydro-
scillaren A

Figure 17

ATPase activity of frog retinal outer segments as a function of glycoside concentration. Upper curve, 17-alpha cymarin. Lower curve, cymarin. Each point is mean ± standard error of mean (represented by vertical bar) of three determinations. All assay tubes contained 0.4% (v/v) ethanol. Other contents described under Figure 15. Assay conditions described in text. See also Figure 16 for further explanation of this figure.


100
90
80
70
60
% of maximum ATPase activity
-5
-6
-7
-8
-9
-10
-∞
Log (inhibitor)
Cymarin
17 α Cymarin

hence results of these experiments are plotted as the mean ± standard error of the mean of three or four determinations. It is clear that sigmoid curves, similar to that observed with ouabain (Figure 15), will fit these points, and that cymarin and scillaren A are considerably more effective inhibitors of Na^+-K^+ dependent ATPase than are the other two glycosides.

The third column of Table VI gives the concentrations of these glycosides necessary to inhibit 50% of the Na^+-K^+ dependent ATPase activity in frog outer segments. These numbers are determined from Figures 16 and 17. Relative inhibitory effectiveness of the four glycosides is shown in the fourth column of Table VI. Because of the uncertainty involved in plotting the inhibition curves of Figures 16 and 17, the numbers given in the last two columns of Table VI are only approximate. Nevertheless, examination of Table VI clearly reveals that cymarin and scillaren A are highly effective inhibitors of both the Na^+-K^+ ATPase in frog outer limbs and of electrical activity in the isolated frog retina. 17-alpha cymarin and hexahydroscillaren A, on the other hand, are much less effective in both cases. Although the quantitative agreement is not precise, perhaps because of experimental uncertainty, the correspondence of the relative efficiencies of the four glycosides as ATPase inhibitors and as inhibitors of electrical activity in

the retina suggests strongly that generation of electrical activity in the retina depends on the integrity of the sodium pump in that tissue, and very possibly specifically in the photoreceptor cells themselves.

Discussion

I. Enzymatic experiments on pig retinal tissue

Comparison of outer segment ATPase activity with similar preparations from other sources: The enzyme system described in this paper is similar to ouabain-sensitive ATPase preparations from a wide variety of sources with respect to cation requirements (Skou, 1957; Post, et al., 1960; Dunham and Glynn, 1961; Wheeler and Whittam, 1962), pH optimum (Skou, 1957; Järnefelt, 1961; Dunham and Glynn, 1961; Wheeler and Whittam, 1962; Auditore and Murray, 1962; Taylor, 1962), sensitivity to ouabain (Post et al., 1960; Bonting et al., 1961), and inhibition by calcium ions (Skou, 1957). The inhibitory effect of high potassium that can be largely reversed by elevated sodium concentrations also has been observed in other preparations (Skou, 1957; Post et al., 1960; Wheeler and Whittam, 1962). Possibly at high concentrations, potassium, which is required for hydrolysis of a phosphorylated intermediate (Charnock and Post, 1963b; Charnock et al., 1963), also competes for attachment at a sodium binding site (Post et al., 1960; Wheeler and Whittam, 1962).

Some differences, however, have been noted. Unlike the preparation from crab nerve (Skou, 1957), the outer segments appear to hydrolyze ADP at a moderate rate. However, whether this observation indicates direct hydrolysis

of ADP or the presence of adenylate kinase has not been determined. Erythrocyte ATPase, which is said to hydrolyze ADP, does not attack ITP (Post et al., 1960). The sodium-potassium activated preparation from rat brain microsomes hydrolyzes GTP, ITP, and UTP at virtually the same rate as it does ATP (Järnefelt, 1962).

Relation to rhodopsin: The similarities with red cell ghosts suggest that rod outer segments contain an ATPase system which may function in the coupled transport of sodium and potassium. In the face of what appears to be a widespread distribution of this enzyme system, it would be surprising if in the photoreceptor outer segment it were singularly specialized to respond to light.

The present results, and those of Bonting et al. (1964), indicate that, in contrast to the suggestion of McConnell and Scarpelli (1963), the ATPase activity of outer limbs should not be attributed to rhodopsin. Bonting et al. have reported that light, which should have bleached all the rhodopsin present in their preparations, had no effect on either the Na^{+}-K^{+}-Mg^{++} sensitive component or the Mg^{++}-sensitive component of outer segment ATPase activity in cattle and frog retinas. Similarly, the addition of hydroxylamine in the light served neither to activate nor to inhibit either component of enzyme activity. The present results are in agreement with these authors that light has no effect

on retinal outer segment ATPase, with or without hydroxylamine. In preparations from pig eyes, the K_m and V_{max} of the total enzyme activity (both components measured together) were the same whether the incubations were carried out in darkness or in bright white light.

In agreement with those of other workers (McConnell and Scarpelli, 1963; Bonting et al., 1964), the present results show that digitonin extracts some ATPase activity as well as rhodopsin from outer segments. Pretreating with alum before digitonin extraction destroys ATPase activity (D. G. McConnell, personal communication), although it is known that such treatment does not affect rhodopsin. Similarly, CTAC extracts rhodopsin from rod outer segments, but very little ATPase activity can be detected, either in the extract, the washed sediment, or uncentrifuged CTAC-containing suspensions. These experiments suggest that the ATPase(s) and rhodopsin are different proteins. Such results, however, could conceivably be interpreted as indicating that sites on the rhodopsin molecule required for enzymatic activity are more labile than other sites involved in interaction with the chromophore. What is needed to eliminate this possibility is evidence that rhodopsin and ATPase activity can be separated. Bonting et al. (1964) showed that the distribution of ATPase activity in fractionated retinas cannot be correlated with rhodopsin, and the present finding that ATPase activity per unit weight

of tissue is approximately the same elsewhere in the retina as it is in the outer segments is consistent with this observation. In addition, the present experiments show that, after a treatment with digitonin that removes all the rhodopsin from the outer segments, ATPase activity can be detected in both the extract and the residue. Such differential solubility is further evidence that the ATPase activity of the rod outer segments is not a property of the visual pigment.

McConnell (1965) has criticized these conclusions, which have previously been published (Frank and Goldsmith, 1965). He claims that significant mitochondrial contamination of these preparations casts doubt on the conclusion that ATPase activity in the outer segments is distinct from rhodopsin because it can be partially separated from the visual pigment by digitonin extraction (Table V). The implication is that the ATPase activity in the sediment after digitonin extraction is mitochondrial in origin, while that in the supernatant is not entirely so. It should first of all be strongly emphasized that the argument that rhodopsin and the outer segment ATPase system are separate, unrelated entities rests on several experimental results (see also Bonting et al., 1964), and not on this one alone. Moreover, examination of Table V clearly reveals that more than 33% of the activity in the sediment is inhibited by ouabain, and hence cannot be a

mitochondrial ATPase. These data (Tables I-V and Figures 2, 3, 4, and 6) were presented previously (Frank and Goldsmith, 1965) along with pertinent discussion, hence, McConnell's objection seems unwarranted.*

Digitonin and CTAC truly inhibit ATPase activity, for control suspensions of rod outer segments incubated with these substances show greatly decreased activity. The decreased activity in the sediment and supernatant from such extracts cannot therefore have been caused by a loosening of the ATPase system from the membrane site by digitonin and CTAC, followed by solubilization of the enzyme system in the wash liquid. The activity in the control suspension is in fact somewhat less than the sum of that in the supernatant and the sediment, possibly because the sediment, washed free of digitonin, is no longer inhibited and thus shows more activity than it does as part of the control suspension. Although other mechanisms of inhibition are not excluded, it is possible that the ATPase activity requires an organization of molecules in the membranes

*Statistical analysis of the data in Tables III and V confirms that mitochondrial ATPase is neither activated by sodium and potassium nor inhibited by ouabain, but that the ATPase activity remaining in the sediment after digitonin extraction does contain a significant ouabain-sensitive component. Application of the t-test (Handbook of Chemistry and Physics, 1964) to the data for ATPase activity of mitochondria in the presence and absence of ouabain (Table III) yields a P value of approximately 0.1. Hence, the difference is not statistically significant. On the other hand, the difference between the ATPase activities of the sediment after digitonin extraction in the presence and absence of ouabain is statistically highly significant, yielding P much less than 0.01.

of the outer segments which is disrupted by digitonin and CTAC.

One or two enzyme systems?: There is evidence that the ouabain sensitive and ouabain-insensitive components are two separate enzyme systems. Skou (1962) has reported a decrease in the magnesium-activated component (ouabain-insensitive component) without affecting the magnesium-sodium-potassium-activated component (ouabain-sensitive component) by treatment with 0.1% desoxycholate and versene followed by differential centrifugation. Similarly, these experiments show that digitonin inhibition of the two components is unequal (Table V). In addition, the substrate specificities of the two components are not the same (Table I). Finally, the pH-dependence curves of the ouabain-sensitive and ouabain-insensitive components of the pig outer segment preparation are not identical, although the difference is not as great as reported by Bonting et al. (1963) with a preparation from another source.

II. Electrophysiological Experiments on Isolated Frog Retinas

The results of these experiments strongly suggest that the sodium-potassium active transport ATPase system in the vertebrate retina is essential for maintenance of the elec-

trical response to light. Several troublesome points must be discussed, however, before this conclusion can be accepted without reservation.

Inhibitory effects of ethanol vs. inhibitory effects of cardiac glycosides: 4% ethanol, the concentration used in the electrophysiological experiments, significantly inhibits outer segment ATPase activity in vitro, and yet it potentiates the b-wave. On the other hand, cardiac glycosides, which also inhibit ATPase activity, abolish electrical activity in the retina. This seeming paradox at first appears to suggest that the effects of glycosides on electrical activity in the isolated retina must be related to some property other than inhibition of the active transport of sodium and potassium.

It should be clear, however, that the inhibitory effect of ethanol on an enzyme system in vitro may not relate at all to the mode of action of ethanol when it is applied to the intact retina. Ethanol presumably denatures proteins in solution by lowering the dielectric constant of the medium, thus favoring the electrostatic interaction of charged groups on different molecules or on the same molecule (Fruton and Simmonds, 1959). This effect will be maximal when a protein is in solution or in cell-free suspension, so that Brownian movement of particles and rotation of peptide chains which are relatively

unhindered by a rigid cell membrane structure are enhanced. This condition appears to be met better in the enzymatic assays than in the electrophysiological experiments, since after lyophilization and homogenization of the cells, membrane structure would likely be broken down and protein components of the membrane ought to be more free to engage in inter- and intramolecular interactions. In addition, with the cell broken down and the membrane fragments presumably more permeable to ethanol, protein components of the membrane might become available to attack by ethanol that were relatively protected in the intact cell. If such a protein moiety were a part of the ATPase system, and were highly sensitive to ethanol denaturation, this could explain the differing *in vivo* and *in vitro* effects of ethanol.

Evidence for the specificity of cardiac glycoside action: Present experimental evidence suggests that cardiac glycosides in the concentrations used in these experiments may act specifically to block the active transport system for sodium and potassium. Schatzmann (1953) demonstrated some years ago that certain cardiac glycosides and their aglycones inhibited the active, linked transport of sodium and potassium in the red blood cell membrane, but did not affect glycolytic or respiratory enzyme systems. Birks (1962) examined under the electron

microscope cat cervical sympathetic ganglia and frog myoneural junctions which had been perfused with solutions containing approximately 10^{-6}M digoxin over a three-hour period. He found that the nerve cells underwent significant swelling when so treated. The mitochondria shrank and became more dense in appearance. Nissl substance swelled. In myoneural juntions, the motor nerve endings swelled and the number of synaptic vesicles decreased. The remaining vesicles appeared swollen. Muscle cells were not affected by this treatment. Birks suggested that inhibition of the sodium pump by the glycoside permitted sodium and chloride ions to diffuse into the cell, accompanied by water to preserve isosmolarity. Evidence for this specific mechanism is that substitution of sulfate ions, which are less diffusible through membranes, for chloride prevented cellular swelling but did not prevent the subcellular changes. Substitution of isosmolar sucrose for sodium chloride in the perfusing medium prevented all the histologic changes apparently caused by the cardiac glycoside.

Quantitative comparison of ouabain effects on electrical activity and outer segment ATPase: Another troublesome point is that concentrations of ouabain less than 10^{-6}M have no consistent effect in inhibiting electrical activity in the retina, yet 10^{-7}M ouabain inhibits 50% of

the transport ATPase system (Figure 15). Viewed somewhat differently, decreasing the ouabain concentration from 10^{-3}M to 10^{-6}M very significantly diminishes the rate of inhibition of electrical activity in the retina (Figure 14), but there is little difference in the *in vitro* ATPase inhibition at these concentrations (Figure 15).

There are several possible explanations for this point. The ATPase system assayed was in the outer segments. Possibly the site of action of ouabain which produces the observed electrical effects is proximal to the outer segments, at a site where the ATPase system has different properties. Although the sensitivity to ouabain of the ATPase system in other layers of the frog retina has not been tested, data from the pig (Table II) suggests that there is no reason to suspect significant differences in the properties of the enzyme system in the inner retinal layers.

Another explanation is that the *in vitro* sensitivity of the outer segment ATPase system to ouabain is greater than the sensitivity *in vivo*. While this possibility has not been ruled out, it appears unlikely, since there is no evidence to suggest that, in other tissues at least, ouabain inhibition of the transport system is more effective *in vitro* than *in vivo*.

An explanation that seems most likely is, that the

photoreceptors contain a considerable excess of the transport ATPase system for maintenance of sodium and potassium gradients in the dark. Under the conditions of the present experiments, it may be possible to inhibit more than 50% of the available sodium-potassium transport system without diminishing the ionic gradients necessary to maintain electrical excitability in the dark. As inhibition of the "pump" increases much beyond this level, the reserve of the system is eliminated. Further inhibition can now be expected rapidly to diminish electrical excitability, and the rate of decrease may be greatly accelerated even when the inhibition of ATPase activity is increased by only a small fraction. This interpretation applies only to the present experimental conditions, in which relatively dark-adapted retinas were used. When the capacity of the transport system is stressed, as in prolonged and/or high-intensity light adaptation, even slight degrees of inhibition of sodium-potassium transport may have marked electrical effects.

Action of ouabain on electrical activity in other neural tissues: Caldwell and Keynes (1959) applied 10^{-5}M ouabain to the external surface of a squid giant axon. They found that active extrusion of sodium was rapidly inhibited, but there was no effect on the resting or action potential. Ouabain injected intracellularly had no effect

on active transport or electrical activity. The duration of perfusion with ouabain was not noted. How is it possible to reconcile this result with the very different electrical effects of glycosides on the isolated frog retina?

The most likely answer would seem to be that the size of the structures involved is very different in the two cases. Ritchie and Straub (1957) applied 1.5×10^{-6}M ouabain to the isolated rabbit cervical sympathetic trunk and found that this procedure abolished post-tetanic hyperpolarization. When the neural sheath was present, the membrane potential was also abolished, presumably because potassium which leaked out from the axons was retained by the sheath in high concentrations outside the neural membrane.

Ritchie and Straub explained the difference between their results and those obtained with squid axons by noting that the intracellular volume of cervical sympathetic axons in the rabbit is very much smaller than the intracellular volume of the squid giant axon. Hence, leakage of small amounts of sodium into these cells and potassium out could change the intracellular concentration and trans-membrane gradients of these ions much more rapidly than transfer of an equal number of ions in a giant axon. Similarly, if a structure of large membrane surface area and small internal volume in the retina were attacked by ouabain, the electrical effects could be very rapid. There are several possibilities for such a structure. Perhaps the most likely candidates, both because of their small size and ready availability to

substances applied to the receptor surface of the retina, are the membranous discs stacked atop one another within the outer segments of the photoreceptors, and the connecting cilium between the outer segment and the inner segment of the rod and cone cells. That the discs contain an aqueous phase with dissolved ions, and that they are osmotically sensitive, is suggested by electron micrographs (DeRobertis and Lasansky, 1961; Dartnall, 1962) that show swelling of the discs with a large intra-disc space when the tissue is fixed in hypotonic media. The discs have a very large membranous surface area, approximately 57 μ^2 in frog rods, calculated from the data of Wolken (1958), and a very small internal volume (roughly 0.15-0.30 μ^3, also calculated from Wolken's data). The connecting cilium also has a very small internal volume, being a tubular structure with a length, in rabbit rods, of about 0.7 μ and an average diameter of 0.25 μ (DeRobertis, 1956).

One further neurophysiological study utilizing cardiac glycosides should be mentioned for completeness. Birks (1963) applied digoxin at the neuromuscular junction of the frog and found that, after a period of time, there occurred a generalized spontaneous twitching of the muscle, followed by transmission block of all neural impulses across the junction. The twitching was presumably caused

by release of acetylcholine, and both this and the nerve block presumably resulted from accumulation of sodium in the cytoplasm of the presynaptic nerve terminals following inhibition of the Na^+-K^+ pump by digoxin. When the digoxin concentration was approximately 10^{-5}M, twitching occurred after roughly one hour and neuromuscular block occurred somewhat later. Birks states that the minimum time for the onset of twitching after application of the maximum concentration of digoxin was 30 minutes, however he does not state what was the maximal digoxin concentration he used. By comparison with the present experiments, it should be noted that the ERG in the isolated frog retina was abolished 20 minutes after application of 10^{-5}M ouabain, and 5 minutes after application of 10^{-3}M glycoside. One obvious explanation for this difference is, once again, that the structure involved in the retina has a smaller internal volume than the presynaptic nerve terminals in Birks' experiments. The latter structures, from Birks' earlier electron micrographs (Birks, 1962), have a diameter of more than 1 μ, and are at least 1 μ long. This clearly assigns to them a much larger intracellular volume than the discs of the rod and cone outer segments, or the connecting cilium.

III. Conclusion: Some Speculations on the Mechanism of Visual Excitation

How a light photon of the appropriate energy, striking the retina, can initiate a neural impulse that is perceived as vision by the brain is a still incompletely answered question that has long been of central importance to visual physiologists. That rhodopsin and the other rod and cone pigments are critical to the excitation process is well known (Wald, 1959), but precisely what is their biological function, is as yet uncertain. Still another intriguing problem is how the light stimulus is amplified. Since the work of Hecht, Shlaer, and Pirenne (1941-42) it has been recognized that as little as one photon of the appropriate energy can excite a single rod. This is such a tiny stimulus that the visual system must clearly be capable of tremendous amplification. The remainder of this paper is devoted to a speculative discussion of these questions, with particular reference to the results reported here.

Much of the speculative discussion of the mechanism of visual excitation has come from George Wald (1954a, 1954b, 1956, 1961, 1965; Wald, Brown, and Gibbons, 1963), whose contributions to this field of research have been extensive. The two alternative models which have been

most prominent in Wald's writings are: (i) the visual pigments are inactive forms of enzymes, much like the zymogens of the upper digestive tract. Light activates these enzymes and thereby triggers the visual process. If the visual pigments are enzymes with high "turnover numbers," and they in turn activate other enzymes, which may activate still other enzymes, one can see how considerable amplification of the initial stimulus can be achieved. Wald (1965) has recently discussed this possibility, using as analogy the multi-enzyme "cascade" presumed to operate in the blood clotting system. (ii) Light "opens a hole" in some fashion in the receptor membrane, thereby triggering the visual mechanism. The "hole" may be (as I believe it is) an increase in membrane permeability to ions, allowing a depolarization by alteration of trans-membrane ionic gradients (see Brindley, 1960; Wald, 1961; Bonting et al., 1964), or it may involve a "radiationless transfer" of energy, perhaps by a current of electrons in the solid state (Tollin et al., 1958; Hagins and Jennings, 1959; Wald, 1961).

Other theories of visual excitation appear much less likely. Fuortes (1959) suggested, based on electrophysiological studies in the eye of the horseshoe crab, Limulus, that the immediate effect of light on the photoreceptor cell is to release a chemical neurotransmitter which then depolarizes the second-order neuron. It is unlikely, how-

ever, that this can be the primary visual event in the vertebrate photoreceptor cell, occurring distally in the outer segment. The latencies of the electrical responses are so brief--1.5 msec. minimum for the a-wave (Brown and Murakami, 1964a) and 20 msec. minimum for the b-wave (Cone, 1964b)--that diffusion of a transmitter agent over the 50-100 μ distance from the distal outer segment to the postsynaptic second-order membrane seems improbable (Brindley, 1960; Hagins et al., 1962). Release of a neurotransmitter at the presynaptic membrane in the photoreceptor cells may, however, occur as a terminal excitatory event in those cells, after rapid initial events take place distally in the outer segment following the light stimulus to trigger the release of the transmitter agent. Such a possibility is suggested by the results of Cone (1964c) to be responsible for the initiation of the b-wave in the bipolar cells. In this case, the diffusion distance is clearly very small.

Other proposed mechanisms have been developed in much less detail. Sjöstrand (1961) has suggested that the initial photochemical event in the outer segment may be amplified by an as yet unknown means in the inner segment. McConnell (1965), struck by apparent anatomical similarities between such layered structures as mitochondria and chloroplasts and the photoreceptor outer segments, states that visual excitation in the rods and cones may "have

certain enzymic principles in common with the energy-transforming systems" of these other organelles. It will not be possible to discuss these two proposals until they have been presented in more detail.

Wald's suggestion that the effect of light is to activate an enzyme, thereby triggering a multi-enzyme "cascade" in the photoreceptor cell, is an interesting idea which is as yet supported by no experimental evidence. The number of enzymes presently known to be located in the photoreceptor outer segment is small, but this does not mean that other enzymes may not be discovered which could function in such a light-activated amplification system.

The results presented in this paper, as well as some other experimental evidence which will be cited, may be interpreted to support what may be called the "membrane hole" theory of visual excitation. As previously noted, Wald has suggested that light quanta could initiate either a membrane permeability change, allowing for ionic fluxes through some part of the photoreceptor, or there could result a radiationless transfer of energy in the form of electrons or "excitons" in the solid state. Such energy transfer presumably occurs following light absorption in plant chloroplasts (Tollin et al., 1958). However, as pointed out by Wald (1961), the processes of vision and photosynthesis are chemically very different even though

light energy is involved in both. In fact, the experiments of Hagins and Jennings (1959) strongly suggest that radiationless energy transfer does not play a part in visual excitation.

The possibility that light excites the photoreceptor by altering the ionic permeability at the site in the disc membrane that absorbs a photon of the appropriate energy seems to be a very plausible mode of visual excitation. There is a clear precedent for neural excitation on the basis of altered sodium and potassium permeabilities, at least in peripheral axons, in the brilliant work of Hodgkin and Huxley and their co-workers (Hodgkin, 1958). That sodium and potassium are necessary for electrical excitability in the retina is clear from the experiments of Hamasaki (1963). Using isolated frog retina preparations similar to those employed in the present work, he showed that the ERG disappeared rapidly when lithium or choline were substituted for sodium in the Ringer's solution bathing the retina. That the effect was principally on the rods and cones and bipolar cells was suggested by the more rapid abolition of the ERG when the sodium-free solutions were applied to the receptor surface than to the vitreous surface of the retina, as well as by the evidence, already presented, that all of the components of the ERG except for the c-wave arise in these cells. Hamasaki also demonstrated that there was a slow fall in the amplitude

of the ERG when the retina was incubated in potassium-free Ringer. It is well known that application of very high concentrations of potassium to the retina abolishes the ERG rapidly, presumably by depolarizing the sensitive cells (Granit, 1962b; Hamasaki, 1964). All of these effects are completely reversible when the retina is returned to normal Ringer (Hamasaki, 1963, 1964).

The present experiments suggest that there is an active sodium pump in the photoreceptor outer segments, since they demonstrate the presence in these organelles of a very high concentration of the enzyme system that has been so strongly implicated in the sodium-potassium active transport system. The electrophysiological experiments on isolated frog retinas suggest that this transport system is essential for maintenance of the electrical response to light. If light "opens a hole" for sodium and, perhaps, potassium in the disc membranes, these ions could diffuse down their gradients and cause electrical activity to arise in the outer segments. The discs seem ideally suited for such a function, because their shape gives them a maximal surface area for light absorption and ionic diffusion, and a minimal internal volume. Hence, a relatively small shift of ions could markedly alter the ionic concentrations inside the disc as well as the trans-membrane ionic gradients.

The effect of glycosides in the present experiments

would appear simply to be the inhibition of the active transport of sodium and potassium so that these ions can leak through the disc membranes down their gradients in the dark and, more rapidly, in the light until the cell is no longer excitable. Presumably this occurs first in the proximal discs, just beyond the connecting cilium, since at least in several species examined with the electron microscope (Cohen, 1960, 1963) the proximal discs have a larger portion of their circumference contained in the plasma membrane bounding the rod, in direct contact with the glycoside-containing extracellular fluid. If the P III component of the ERG originates in the outer segment and spreads proximally by electrotonic conduction through the plasma membrane to the proximal synaptic terminal, there to initiate the b-wave in the bipolar cell, one can see why a conduction block in the proximal outer segment would eliminate the b-wave and preserve P III. Of course, if the block occurs in the connecting cilium, a similar effect would be observed. (Note that this interpretation differs from the origin of electrical activity in the photoreceptor cell proposed by Brown and Murakami (1964a, 1964b) and discussed earlier.) The more distal discs appear to have less of their circumference in contact with the extracellular fluid, and some appear to be entirely enclosed by the plasma membrane (Cohen, 1960). Hence, these discs should take longer to depolarize--some

probably even requiring the glycoside to permeate the plasma membrane in order to block transport sites--and P III should be preserved until all the discs are inactivated.

This model suggests that, in the light, the sodium pump will work harder than in darkness in an attempt to maintain the trans-membrane ionic gradients and, hence, excitability in the face of increased passive ionic fluxes. This is not contradictory to the evidence presented here, that light does not increase ATPase activity in outer segments, since the present experiments involved outer segments exposed to light while suspended in media of constant ionic composition, which contacts both the inner and the outer surface of the membranes of the now disrupted photoreceptor. In vivo, however, light initiates changes in the ionic composition on either side of the disc membrane. The enzymatic data here clearly show that enzymatic activity is strongly influenced by the concentrations of sodium and potassium present, and it is these changes induced by light, and not the light itself, that should increase pump activity. That ATPase activity in the rods is increased in the light is indirectly suggested by two pieces of experimental evidence. Auricchio and DeBerardinis (1952) found that levels of high-energy phosphate esters in the retina decreased in the light. Enoch (1963, 1964) showed increased staining of the distal inner

segments with nitro-blue tetrazolium in a histochemical method for detection of succinoxidase activity in light-adapted retinas over those which were kept in the dark. This suggests increased activity of the tricarboxylic acid cycle in the light in the mitochondria of the distal inner segments. If ATP was added to the histochemical incubation medium, the staining decreased both in the light and in the dark (Enoch, 1963). The action spectrum for this reaction closely approximated the rhodopsin absorption spectrum (Enoch, 1964). These experiments imply that more ATP is required in the photoreceptors in the light, and that more is hydrolyzed.

Finally, it is possible to suggest a way in which a light photon striking a visual pigment molecule might alter the ionic permeability of the disc membrane. The following model for the action of light on rhodopsin has been suggested (Kropf and Hubbard, 1958; Hubbard and Kropf, 1959). Light initially stereoisomerizes the retinal chromophore from the 11-*cis* to the all-*trans* form. This "unlocks" the opsin protein so that it is free to alter its secondary and tertiary structure. Finally, the retinal chromophore is hydrolyzed from its Schiff base linkage to the protein, this last step known as "bleaching" because of the large color change which occurs in the solution at this time. Kropf and Hubbard (1958) have suggested that visual excitation is initiated between the first (stereo-

isomerization) step, which produces lumirhodopsin, and the second (protein structural rearrangement), which produces metarhodopsin. The reasons for this are that the final, "bleaching" step is too slow to be important in visual excitation in vertebrate eyes, and in many invertebrates it does not occur at all (Hubbard and St. George, 1956; Wald and Hubbard, 1957; Brown and Brown, 1958). While more recent work on the visual cycle (Yoshizawa and Wald, 1963; Matthews et al., 1963) has demonstrated that the chain of reactions initiated by light on the rhodopsin molecule may be more complex than Hubbard and Kropf's original model, the basic elements of the scheme still seem eminently reasonable. Since rhodopsin is such an important structural element in the rod outer segment (Hubbard, 1954b), a change in its configuration induced by light could profoundly alter the structure of the membrane, perhaps opening actual holes through which ions could freely diffuse. One need not postulate enzymatic activity for the visual pigments at all.

Summary and Conclusions

1. Isolated, lyophilized photoreceptor outer segments of pig and frog retinas contain ATPase activity. At pH 7.2 in the presence of 60 mM Na^+, 20 mM K^+, 3 mM Mg^{++}, and 3 mM ATP the total activity in pig outer segments is approximately 5.00 millimoles phosphate released/gram dry weight of tissue/30 minutes at 37°C. Under the same conditions, the total activity in frog outer segments is about half of this.
2. About 50% of the total ATPase activity in pig outer segments and about 33% in frogs requires sodium and potassium together in addition to magnesium and is inhibited by cardiac glycosides. Many studies have implicated this enzyme system in the active, linked transport of sodium and potassium across cell membranes. The concentration of ouabain for half-maximal ATPase inhibition is 6.3×10^{-7}M in pig outer segments and 1.0×10^{-7}M in frogs.
3. Further characterization of the enzyme system was carried out in pig retinas. The following results were obtained:
    a) The optimal Mg^{++} concentration is approximately equal to the concentration of ATP.
    b) Optimal concentrations of Na^+ and K^+ are about 15 mM Na^+ and 6 mM K^+. Further increases in K^+ are inhibitory, but this inhibition can be par-

tially overcome by raising the Na^+ concentration. In the presence of Mg^{++}, Na^+ alone stimulates slightly; K^+ alone has no effect.

c) ATP has considerably more activity as a substrate than eight other phosphate esters tested, both in the presence and absence of ouabain.

d) The pH-activity curve has a rather broad maximum with a peak at pH 7.2. Ouabain broadens the maximum and alters the shape of the curve, but does not displace the peak.

e) In addition to ouabain, PCMB and Ca^{++} are also inhibitory. DNP has no effect.

f) The following evidence strongly suggests that the ATPase activity of the photoreceptor outer segments is not a property of the visual pigments:

i. The rate of hydrolysis of ATP by dark-adapted preparations is the same in light as it is in darkness, whether or not hydroxylamine or ouabain is present.

ii. The ATPase activity of pig retinas, exclusive of outer segments, is roughly the same, per unit dry weight, as that of the outer segments alone.

iii. By treatment of dark-adapted outer seg-

ments with digitonin, it is possible to effect a partial separation of rhodopsin and Na^+-K^+ dependent ATPase activity. This activity is not a property of mitochondria, since mitochondrial ATPase is insensitive to sodium and potassium and to ouabain.

iv. CTAC extracts rhodopsin from outer segments but destroys all the ATPase activity in both the extract and the residue.

4. **The electrical activity of isolated frog retinas was studied after treatment with cardiac glycosides.** The following results were obtained:

a) Ouabain suppresses electrical activity. When 10^{-3}M ouabain is applied to the photoreceptor surface of the retina, the b-wave of the ERG disappears in 1-2 minutes and the P III component is abolished in 5 minutes. These effects are irreversible. As the ouabain concentration is decreased, the time course is greatly slowed. At the same concentrations, the time course is also slowed when ouabain is applied to the vitreal surface of the retina.

b) The lowest ouabain concentration to abolish electrical activity is 10^{-6}M. 10^{-7}M (which inhibits

50% of the transport ATPase activity *in vitro*) and lower concentrations have no effect.

c) Scillaren A and cymarin, glycosides which have comparable ATPase-inhibitory activity to ouabain, also are comparable in their ability to abolish electrical activity in the frog retina.

d) Hexahydroscillaren A and 17-alpha cymarin are glycosides with very slight structural differences from scillaren A and cymarin. They are, however, significantly less effective as inhibitors of ATPase activity and sodium-potassium transport in human erythrocyte membranes and of ATPase activity in frog retinal outer segments. Their inhibitory effects on electrical activity in the isolated frog retina are also significantly less than that of scillaren A and cymarin in equal concentrations. The relative inhibitory effects of these four glycosides tested in ATPase assays and electrophysiological experiments are similar. For these reasons it is suggested that cardiac glycosides most likely inhibit electrical activity in the retina because of their inhibition of the enzyme system which mediates active sodium and potassium transport. It is further suggested that this enzyme system is necessary to maintain the

electrical response to light in the retina.

5. The implications of these results are discussed with respect to various theories of the mechanism of visual excitation. It is suggested that light may initiate the visual impulse by creating configurational changes in the visual pigment molecules which are critical components of the photoreceptor outer segment membrane structure. These changes may markedly increase membrane permeability to sodium and/or potassium ions, thus leading to electrical activity in the membrane structures of the outer segments.

References

Adrian, E. D. and Matthews, R. (1927), "The action of light on the eye. Part I. The discharge of impulses in the optic nerve and its relation to the electric changes in the retina," J. Physiol., 63, 378-414.

Aldridge, W. N. (1962), "Adenosine triphosphatase in the microsomal fraction from rat brain," Biochem. J., 83, 527-533.

Anfinsen, C. B. (1944), "The distribution of cholinesterase in the bovine retina," J. Biol. Chem., 152, 267-278.

Auditore, J. V. and Murray, L. (1962), "Cardiac (microsomal) Na plus K adenosinetriphosphatase and its possible relationship to the active Na plus K transport system," Arch. Biochem. Biophys., 99, 372-382.

Auricchio, G. and DeBerardinis, E. (1951) "Influenza della illuminazione sull'attivita adenilpirofosfatasica della retina," Boll. Soc. ital. biol. sper. Nap., 27, 71-73.

Auricchio, G. and DeBerardinis, E. (1952), "Ciclo dei fosfati nella retina e suo significato biologico," Ann. Ottalm., 78, 53-60.

Berkow, J. W. and Patz, A. (1961), "Histochemistry of the retina. II. Use of phenazine methosulfate to demonstrate the succinoxidase system," A.M.A. Arch. Ophthalmol., 65, 828-831.

Bernhard, C. G. and Skoglund, C. R. (1941), "Selective suppression with ethylalcohol of inhibition in the optic nerve and of the negative component P III of the electroretinogram," Acta Physiol. Scand., 2, 10-21.

Birks, R. I. (1962), "The effects of a cardiac glycoside on subcellular structures within nerve cells and their processes in sympathetic ganglia and skeletal muscle," Canad. J. Biochem. Physiol., 40, 303-316.

Birks, R. I. (1963), "The role of sodium ions in the metabolism of acetylcholine," Canad. J. Biochem. Physiol., 41, 2573-2597.

Bonting, S. L., Simon, K. A., and Hawkins, N. M. (1961), "Studies on sodium-potassium activated adenosine triphosphatase. I. Quantitative distribution in several tissues of the cat," Arch. Biochem. Biophys., 95, 416-423.

Bonting, S. L., Caravaggio, L. L., and Hawkins, N. M. (1963), "Studies on sodium-potassium activated adenosine triphosphatase. VI. Its role in cation transport in the lens of cat, calf, and rabbit," Arch. Biochem. Biophys., 101, 47-55.

Bonting, S. L., Caravaggio, L. L., and Canady, M. R. (1964), "Studies on sodium-potassium activated adenosine triphosphatase. X. Occurrence in retinal rods and relation to rhodopsin," Exp. Eye Res., 3, 47-56.

Brindley, G. S. (1960), Chapter II, "The electrical activity of the retina" in Physiology of the Retina and Visual Pathway, London: Edward Arnold, Ltd., pp. 43-67.

Brown, K. T. and Murakami, M. (1964a), "A new receptor potential of the monkey retina with no detectable latency," Nature, 201, 626-628.

Brown, K. T. and Murakami, M. (1964b), "Biphasic form of the early receptor potential of the monkey retina," Nature, 204, 739-740.

Brown, K. T. and Watanabe, K. (1962a), "Isolation and identification of a receptor potential from the pure cone fovea of the monkey retina," Nature, 193, 958-960.

Brown, K. T. and Watanabe, K. (1962b), "Rod receptor potential from the retina of the night monkey," Nature, 196, 547-550.

Brown, K. T. and Wiesel, T. N. (1961a), "Localization of origins of electroretinogram components by intraretinal recording in the intact cat eye," J. Physiol., 158, 257-280.

Brown, K. T. and Wiesel, T. N. (1961b), "Analysis of the intraretinal electroretinogram in the intact cat eye," J. Physiol., 158, 229-256.

Brown, P. K. and Brown, P. S. (1958), "Visual pigments of the octopus and cuttlefish," Nature, 182, 1288-1290.

Byzov, A. L. (1959), "Analysis of the distribution of potentials and currents within the retina on light stimulation. I. Activity of bipolars of two types," Biophys., Lond., 4, 46-59.

Byzov, A. L. (1964), "Origin and some properties of component P III of the frog electroretinogram," Fed. Proc.

(Translation suppl.), 23, 230-234. Russian original in Fiziol. Zhurn. SSSR, 49, 440 (1963).

Caldwell, P. C. and Keynes, R. D. (1959), "The effect of ouabain on the efflux of sodium from a squid giant axon," J. Physiol., 148, 8P-9P.

Charnock, J. S. and Post, R. L. (1963a), "Studies of the mechanism of cation transport. I. The preparation and properties of a cation-stimulated adenosine-triphosphatase from guinea pig kidney cortex," Aust. J. Exp. Biol. Med. Sci., 41, 547-560.

Charnock, J. S. and Post, R. L. (1963b), "Evidence of the mechanism of ouabain inhibition of cation-activated adenosine triphosphatase," Nature, 199, 910-911.

Charnock, J. S., Rosenthal, A. S., and Post, R. L. (1963), "Studies of the mechanism of cation transport. II. A phosphorylated intermediate in the cation stimulated enzymic hydrolysis of adenosine triphosphate," Aust. J. Exp. Biol. Med. Sci., 41, 675-686.

Cogan, D. G. and Kuwabara, T. (1959), "Tetrazolium studies on the retina. II. Substrate dependent patterns," J. Histochem. Cytochem., 7, 334-341.

Cogan, D. G., Eränkö, O., Sjöstrand, F. S., and Pearse, A. G. E. (1961), Discussion in The Structure of the Eye, G. K. Smelser, ed., New York: Academic Press, pp. 70-72.

Cohen, A. I. (1960), "The ultrastructure of the rods of the mouse retina," Am. J. Anat., 107, 23-48.

Cohen, A. I. (1963), "Vertebrate retinal cells and their organization," Biol. Revs., 38, 427-459.

Cole, D. F. (1964), "Location of ouabain-sensitive adenosine triphosphatase in ciliary epithelium," Exp. Eye Res., 3, 72-75.

Collins, F. D., Love, R. M., and Morton, R. A. (1951), "The preparation of rhodopsin and the chemical composition of rod outer segments," Biochem. J., 48, xxxv.

Collins, F. D., Love, R. M., and Morton, R. A. (1952), "Studies in rhodopsin. 5. Chemical analysis of retinal material," Biochem. J., 51, 669-673,

Commission on Nomenclature of Biological Chemistry (1960), "Definitive rules for the nomenclature of amino acids, steroids, vitamins, and carotenoids," J. Am. Chem. Soc., 82, 5575-5584.

Cone, R. A. (1963), "Quantum relations of the rat ERG," J. Gen. Physiol., 46, 1267-1286.

Cone, R. A. (1964a), "Early receptor potential of the vertebrate retina," Nature, 204, 736-739.

Cone, R. A. (1964b), "The rat electroretinogram. I. Contrasting effects of adaptation on the amplitude and latency of the b-wave," J. Gen. Physiol., 47, 1089-1105.

Cone, R. A. (1964c), "The rat electroretinogram. II. Bloch's law and the latency mechanism of the b-wave," J. Gen. Physiol., 47, 1107-1116.

Dartnall, H. J. A. (1957), Chapter I, "Introduction and Methods" in The Visual Pigments, London: Methuen and Co., Ltd., pp. 1-24.

Dartnall, H. J. A. (1962), "The properties of visual pigments in photoreceptors" in The Eye, H. Davson, ed., Volume 2, The Visual Process, New York: Academic Press, pp. 473-479.

Davson, H. (1964), Chapter X, "Ionic Equilibria, Bioelectric Potentials, and Active Transport," pp. 376-480, and Chapter XV, "Excitability and Propagation of the Impulse," pp. 671-745 in A Textbook of General Physiology, Boston: Little, Brown and Co.

DeBerardinis, E. and Auricchio, G., (1951), "Sull'attivita adenilpirofosfatasica della retina," Boll. Soc. ital. biol. sper. Nap., 27, 68-71.

DeRobertis, E. (1956), "Electron microscope observations on the submicroscopic organization of the retinal rods," J. Biophysic. and Biochem. Cytol., 2, 319-330.

DeRobertis, E. and Lasansky, A. (1961), "Ultrastructure and chemical organization of photoreceptors," in The Structure of the Eye, G. K. Smelser, ed., New York: Academic Press, pp. 29-52.

Deul, D. H. and McIlwain, H. (1961), "Activation and inhibition of adenosine triphosphatases of subcellular particles from the brain," J. Neurochem., 8, 246-256.

Dowling, J. E. (1963), "Neural and photochemical mechanisms of visual adaptation in the rat," J. Gen. Physiol., 46, 1287-1301.

Dunham, E. T. and Glynn, I. M. (1961), "Adenosinetriphosphatase activity and the active movements of alkali metal ions," J. Physiol., 156, 274-293.

Enoch, J. M. (1963), "The use of tetrazolium to distinguish between retinal receptors exposed and not exposed to light," Invest. Ophthalmol., 2, 16-23.

Enoch, J. M. (1964), "Validation of an indicator of mammalian retinal receptor response: action spectrum," J. Opt. Soc. Amer., 54, 368-379.

Eränkö, O., Niemi, M., and Merenmies, E. (1961), "Histochemical observations on esterases and oxidative enzymes of the retina," in The Structure of the Eye, G. K. Smelser, ed., New York: Academic Press, pp. 159-171.

Frank, R. N. and Goldsmith, T. H. (1965), "Adenosine triphosphatase activity in the rod outer segments of the pig's retina," Arch. Biochem. Biophys., 110, 517-525.

Fruton, J. S. and Simmonds, S. (1959), Chapter 2, "General Properties of Proteins," in General Biochemistry, 2nd edition, New York: John Wiley and Sons, Inc., pp. 20-21.

Fuortes, M. G. F. (1959), "Initiation of impulses in visual cells of Limulus," J. Physiol., 148, 14-28.

Futterman, S. (1963), "Metabolism of the retina. III. The role of reduced triphosphopyridine nucleotide in the visual cycle," J. Biol. Chem., 238, 1145-1150.

Glynn, I. M. (1957a), "The ionic permeability of the red cell membrane," Progr. Biophys., 8, 241-307.

Glynn, I. M. (1957b), "The action of cardiac glycosides on sodium and potassium movements in human red cells," J. Physiol., 135, 148-173.

Glynn, I. M. (1964), "The action of cardiac glycosides on ion movements," Pharmacol. Revs., 16, 381-407.

Granit, R. (1959), Chapter XXIX, "Neural activity in the retina" in Handbook of Physiology, Section 1: Neurophysiology, Volume I, H. W. Magoun, ed., Washington, D. C.: The American Physiological Society, pp. 693-712.

Granit, R. (1962a), Chapter 5, "The Electroretinogram," in Receptors and Sensory Perception, New Haven: Yale University Press paperbound edition, pp. 151-190.

Granit, R. (1962b), "Sensory hyperpolarizations" in Receptors and Sensory Perception, New Haven: Yale University Press paperbound edition, pp. 28-32.

Granit, R. and Therman, P. O., (1935), "Excitation and inhibition in the retina and in the optic nerve," J. Physiol., 83, 359-381.

Gray, J. A. B. (1959), Chapter IV, "Initiation of Impulses at Receptors" in Handbook of Physiology, Section 1: Neurophysiology, Volume I, H. W. Magoun, ed., Washington, D. C.: The American Physiological Society, pp. 123-146.

Hagins, W. A. and Jennings, W. H. (1959), "Radiationless migration of electronic excitation in retinal rods," Disc. Faraday Soc., 27, 180-190.

Hagins, W. A., Zonana, H. V., and Adams, R. G. (1962), "Local membrane current in the outer segments of squid photoreceptors," Nature, 194, 844-847.

Hamasaki, D. I. (1963), "The effect of sodium ion concentration on the electroretinogram of the isolated retina of the frog," J. Physiol., 167, 156-168.

Hamasaki, D. I. (1964), "The electroretinogram after application of various substances to the isolated retina," J. Physiol., 173, 449-458.

Handbook of Chemistry and Physics (1964), 45th edition, R. C. Weast, S. M. Selby, and C. D. Hodgman, eds., Cleveland, Ohio: The Chemical Rubber Co., pp. A-109-A-110.

Hecht, S. Shlaer, S. and Pirenne, M. H. (1941-42), "Energy, quanta, and vision," J. Gen. Physiol., 25, 819-840.

Hess, H. H. and Pope, A. (1961), "Intralaminar distribution of adenosinetriphosphatase activity in human frontal isocortex," J. Neurochem., 8, 299-309.

Hodgkin, A. L. (1958), "Ionic movements and electrical activity in giant nerve fibers," Proc. Royal Soc., B-148, 1-37.

Hokin, L. E. and Hokin, M. R. (1960), "Studies on the en-

zymatic mechanism of the sodium pump" in Symposium on Membrane Transport and Metabolism, A. Kleinzeller and A. Kotyk, eds., New York: Academic Press, pp. 204-218.

Hubbard, R. (1954a), "The respiration of the isolated rod outer limb of the frog retina," J. Gen. Physiol., 37, 373-379.

Hubbard, R. (1954b), "The molecular weight of rhodopsin and the nature of the rhodopsin-digitonin complex," J. Gen. Physiol. 37, 381-399.

Hubbard, R. (1956), "Retinene isomerase," J. Gen. Physiol., 39, 935-962.

Hubbard, R. and Kropf, A. (1959), "Molecular aspects of visual excitation," Ann. N.Y. Acad. Sci., 81, 388-398.

Hubbard, R. and St. George, R. C. C. (1956), "The rhodopsin system of the squid," J. Gen. Physiol., 41, 501-528.

Hubbard, R. and Wald, G. (1951), "The mechanism of rhodopsin synthesis," Proc. Nat. Acad. Sci., U.S., 37, 69-79.

Järnefelt, J. (1961), "Sodium-stimulated adenosinetriphosphatase in microsomes from rat brain," Biochim. Biophys. Acta, 48, 104-110.

Järnefelt, J. (1962), "Properties and possible mechanism of the Na-ion and K-ion stimulated microsomal adenosinetriphosphatase," Biochim. Biophys. Acta, 59, 643-654.

Judah, J. D. and Ahmed, K. (1964), "The biochemistry of sodium transport," Biol. Revs., 39, 381-407.

Kinsolving, C. R., Post, R. L., and Beaver, D. L. (1963), "Sodium plus potassium transport adenosine triphosphatase activity in kidney," J. Cell. Comp. Physiol., 62, 85-93.

Kropf, A. and Hubbard, R. (1958), "The mechansim of bleaching rhodopsin," Ann. N.Y. Acad. Sci., 74, 266-280.

Kuwabara, T. and Cogan, D. G. (1960), "Tetrazolium studies on the retina. III. Activities of metabolic intermediates and miscellaneous subatrates," J. Histochem. Cytochem., 8, 214-224.

Lessell, S. and Kuwabara, T. (1964), "Phosphatase histochemistry of the eye," A.M.A. Arch. Ophthalmol., 71, 851-860.

Lowry, O. H., Roberts, N. R., and Lewis, C. (1956), "The quantitative histochemistry of the retina," J. Biol. Chem., 220, 879-892.

Lowry, O. H., Roberts, N. R., Schulz, D. W., Clow, J. E., and Clark, J. R. (1961), "Quantitative histochemistry of the retina. II. Enzymes of glucose metabolism," J. Biol. Chem., 236, 2813-2820.

Matthews, R. G., Hubbard, R., Brown, P. K., and Wald, G. (1963), "Tautomeric forms of metarhodopsin," J. Gen. Physiol., 47, 215-240.

McConnell, D. G. (1965), "The isolation of retinal outer segment fragments," J. Cell Biol., 27, 459-474.

McConnell, D. G. and Scarpelli, D. G. (1963), "Rhodopsin: an enzyme?" Science, 139, 848.

Moody, M. F. and Robertson, J. R. (1960), "The fine structure of some retinal photoreceptors," J. Biophysic. and Biochem. Cytol., 7, 87-92.

Niemi, M. and Merenmies, E. (1961a), "Cytochemical localization of the oxidative enzyme systems in the retina. I. Diaphorases and dehydrogenases," J. Neurochem., 6, 200-205.

Niemi, M. and Merenmies, E. (1961b), "Cytochemical localization of the oxidative enzyme systems in the retina. II. Cytochrome oxidase," J. Neurochem., 6, 206-209.

Noell, W. K. (1953), Studies on the Electrophysiology and the Metabolism of the Retina, Randolph Field, Texas: U.S.A.F. School of Aviation Medicine.

Pak, W. L. and Cone, R. A. (1964), "Isolation and identification of the initial peak of the early receptor potential," Nature, 204, 836-838.

Pearse, A. G. E. (1960), Chapter XVIII, "Oxidases and Peroxidases" in Histochemistry, Boston: Little, Brown and Co., pp. 509-516.

Pearse, A. G. E. (1961), "Localization of oxidative enzymes in rat and chick retina in various physiological conditions," in The Structure of the Eye, G. K. Smelser, ed., New York: Academic Press, pp. 53-70.

Post, R. L., Merritt, C. R., Kinsolving, C. R., and Albright, C. D. (1960), "Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte," J. Biol. Chem., 235, 1796-1802.

Potts, A. M. (1962), "Retinotoxic and choroidotoxic substances," Invest. Ophthalmol., 1, 290-303.

Riley, M. V. (1964), "The sodium=potassium stimulated adenosine triphosphatase of rabbit ciliary epithelium," Exp. Eye Res., 3, 76-84.

Ritchie, J. M. and Straub, R. W. (1957), "The hyperpolarization which follows activity in mammalian non-medullated fibres," J. Physiol., 136, 80-97.

Scarpelli, D. G. and Craig, E. L. (1963), "The fine localization of nucleoside triphosphatase activity in the retina of the frog," J. Cell Biol., 17, 279-288.

Schatzmann, H. J. (1953), "Herzglykoside als Hemmstoffe für den aktiven Kalium- und Natriumtransport durch die Erythrocytenmembran," Helv. Physiol. Acta, 11, 346-354.

Schneider, W. C. (1948), "Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions," J. Biol. Chem., 176, 259-266.

Schwartz, A. (1963), "A Na-K stimulated adenosine triphosphatase in 'microsomal' fractions from rat liver," Biochim. Biophys. Acta, 67, 329-331.

Sekoguti, Y. (1960), "On the ATPase activities in the retina and the outer segments," J. Cell. Comp. Physiol., 56, 129-136.

Sidman, R. L. (1958), "Histochemical studies on photoreceptors," Ann. N.Y. Acad. Sci., 74, 164-181.

Sjöstrand, F. S. (1961), "Electron microscopy of the retina" in The Structure of the Eye, G. K. Smelser, ed. New York: Academic Press, pp. 1-28.

Skou, J. C. (1957), "The influence of some cations on an adenosine triphosphatase from peripheral nerves," Biochim. Biophys. Acta, 23, 394-401.

Skou, J. C. (1960), "Further investigations on a Mg^{++}-ion and Na^{+}-ion activated adenosinetriphosphatase, possibly

related to the active, linked transport of Na^+-ion and K^+-ion across the nerve membrane," Biochim. Biophys. Acta, 42, 6-23.

Skou, J. C. (1962), "Preparation from mammalian brain and kidney of the enzyme system involved in active transport of Na^+ and K^+," Biochim. Biophys. Acta, 58, 314-325.

Skou, J. C. (1965), "Enzymatic basis for active transport of Na^+ and K^+ across cell membranes," Physiol. Revs., 45, 596-617.

Taylor, C. B. (1962), "Cation-stimulation of an ATPase system from the intestinal mucosa of the guinea pig," Biochim. Biophys. Acta, 60, 437-440.

Tollin, G., Sogo, P. B., and Calvin, M. (1958),"Energy transfer in ordered and unordered photochemical systems," Ann. N.Y. Acad. Sci., 74, 310-328.

Tomita, T. (1959), "Study on electrical activities in the retina with penetrating microelectrodes," Proc. XXI Int. Cong. Physiol., pp. 245-248.

Tosteson, D. C., Moulton, R. H., and Blaustein, M. (1960), "An enzymatic basis for the difference in active cation transport in two genetic types of sheep," Fed. Proc., 19, 128.

Ussing, H. H. (1954), "Active transport of inorganic ions," Symp. Soc. Exp. Biol., 8, 407-422.

Van Groningen, H. E. M. and Slater, E. C. (1963), "The effect of oligomycin on the $(Na^+ + K^+)$-activated magnesium ATPase of brain microsomes and erythrocyte membranes," Biochim. Biophys. Acta, 73, 527-530.

Wald, G. (1954a), "Mechanisms of vision" in Nerve Impulse: Transactions of the Fourth Conference, D. Nachmansohn, ed., New York: The Josiah Macy, Jr. Foundation, pp. 11-55.

Wald, G. (1954b), "On the mechanism of the visual threshold and visual excitation," Science, 119, 887-892.

Wald, G. (1956), Chapter 17, "The biochemistry of visual excitation" in Enzymes: Units of Biological Structure and Function, O. H. Gaebler, ed., Henry Ford Hospital International Symposium, New York: Academic Press, pp. 355-367.

Wald, G. (1959), Chapter XXVIII, "The photoreceptor process in vision" in Handbook of Physiology, Section 1: Neurophysiology, Volume I, H. W. Magoun, ed., Washington, D. C.: The American Physiological Society, pp. 671-692.

Wald, G. (1961), "General discussion of retinal structure in relation to the visual process" in The Structure of the Eye, G. K. Smelser, ed., New York: Academic Press, pp. 101-116.

Wald, G. (1965), "Visual excitation and blood clotting," Science, 150, 1028-1030.

Wald, G., Brown, P. K., and Gibbons, I. R. (1963), "The problem of visual excitation," J. Opt. Soc. Amer., 53, 20-35.

Wald, G. and Hubbard, R. (1948-49), "The reduction of retinene$_1$ to vitamin A$_1$ in vitro," J. Gen. Physiol., 32, 367-389.

Wald, G. and Hubbard, R. (1957), "Visual pigment of a decapod crustacean: the lobster," Nature, 180, 278-280.

Wheeler, K. P. and Whittam, R. (1962), "Some properties of a kidney adenosine triphosphatase relevant to active cation transport," Biochem. J., 85, 495-507.

Whittam, R. (1962), "The asymmetrical stimulation of a membrane adenosinetriphosphatase in relation to active cation transport," Biochem. J., 84, 110-118.

Wolken, J. J. (1958), "Studies of photoreceptor structures," Ann. N.Y. Acad. Sci., 74, 164-181.

Wolken, J. J. (1961), "A structural model for a retinal rod" in The Structure of the Eye, G. K. Smelser, ed., New York: Academic Press, pp. 173-191.

Yamashita, E. (1959), "Some analyses of slow potentials of toad's retina," Tokohu J. Exp. Med., 70, 221-233.

Yoshida, H. and Fujisawa, H. (1962), "Influence of subcellular structures on the activity of Na ion, K ion activated adenosine triphosphatase in brain," Biochim. Biophys. Acta, 60, 443-444.

Yoshizawa, T. and Wald, G. (1963), "Pre-lumirhodopsin and the bleaching of visual pigments," Nature, 197, 1279-1286.

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE
M. Heard 1 South	7/2/67